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14. ABSTRACT

Numerous studies have shown that regulated exocytosis is activated simultaneously by calcium, guanosine triphosphate (GTP) and protein kinase C (PKC), and that this process is specifically inhibited by botulinum toxins (BoNTs). Although phenomenologically well known, the specific sites of action for these agents in the late stage of exocytosis, membrane fusion, remain unknown. In this research project, we combined both in vitro and in vivo approaches to directly test the effects of these agents on annexin 7. Annexin 7 (ANX7) is a calcium-dependent GTP-activated membrane fusion protein. In a reconstituted membrane fusion system using artificial liposomes, ANX7 membrane fusion activity is substantially increased by the combination of individually optimal concentration of guanine nucleotide and PKC. This increasing ANX7 activity can be distinguished by a simple additive model when comparing activation by either guanine nucleotide or PKC alone. In other in vitro assays, the binding of GTP and its non-hydrolyzable analogues to ANX7 significantly enhance PKC phosphorylation, and conversely PKC phosphorylation markedly potentiates the binding and hydrolysis of GTP by ANX7. While certain other kinases label ANX7 efficiently, they do not substitute for PKC in potentiating GTP binding or membrane fusion. To correlate the in vitro data with exocytotic events in cells, we examined the biochemical profile of endogenous ANX7 in secreting adrenal chromaffin cells. In vivo, both the ratio of ANX7-bound GDP/GTP as well as ANX7 phosphorylation by PKC change in proportion to the extent of catecholamine release from stimulated chromaffin cells. Thus, the stimulatory actions of calcium, GTP and PKC appear to specifically converge on ANX7 to drive membrane fusion activity occurring during exocytosis. To further support such an inference, we have found that BoNT type C efficiently cleaves ANX7 both in vitro and in permeabilized chromaffin cells. This proteolytic activity is concurrent with BoNT/C-dependent inhibition of ANX7 membrane fusion activity in vitro, and with inhibition of catecholamine secretion in vivo. We therefore conclude that the exocytotic machinery includes ANX7 as a common site of action for calcium, GTP, PKC and Botulinum toxin in the exocytotic membrane fusion process.

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ABSTRACT

MEMBRANE FUSION PROTEIN ANNEXIN 7: A COMMON SITE OF ACTION FOR CALCIUM, GUANOSINE TRIPHOSPHATE, PROTEIN KINASE C AND BOTULINUM TOXIN TYPE C IN REGULATED EXOCYTOSIS

Hung Caohuy

directed by Harvey B. Pollard, M.D., Ph.D., Professor and Chair of Department of Anatomy, Physiology and Genetics

Numerous studies have shown that regulated exocytosis is activated simultaneously by calcium, guanosine triphosphate (GTP) and protein kinase C (PKC), and that this process is specifically inhibited by botulinum toxins (BoNTs). Although phenomenologically well known, the specific sites of action for these agents in the late stage of exocytosis, membrane fusion, remain unknown.

In this research project, we combined both *in vitro* and *in vivo* approaches to directly test the effects of these agents on annexin 7. Annexin 7 (ANX7) is a calcium-dependent GTP-activated membrane fusion protein. In a reconstituted membrane fusion system using artificial liposomes, ANX7 membrane fusion activity is substantially increased by the combination of individually optimal concentration of guanine nucleotide and PKC. This increasing ANX7 activity can be distinguished by a simple additive model when comparing activation by either guanine nucleotide or PKC alone. In other *in vitro* assays, the binding of GTP and its non-hydrolyzable analogues to ANX7 significantly enhance PKC

phosphorylation, and conversely PKC phosphorylation markedly potentiates the binding and hydrolysis of GTP by ANX7. While certain other kinases label ANX7 efficiently, they do not substitute for PKC in potentiating GTP binding or membrane fusion.

To correlate the *in vitro* data with exocytotic events in cells, we examined the biochemical profile of endogenous ANX7 in secreting adrenal chromaffin cells. *In vivo*, both the ratio of ANX7-bound GDP/GTP as well as ANX7 phosphorylation by PKC change in proportion to the extent of catecholamine release from stimulated chromaffin cells. Thus, the stimulatory actions of calcium, GTP and PKC appear to specifically converge on ANX7 to drive membrane fusion activity occurring during exocytosis. To further support such an inference, we have found that BoNT type C efficiently cleaves ANX7 both *in vitro* and in permeabilized chromaffin cells. This proteolytic activity is concurrent with BoNT/C-dependent inhibition of ANX7 membrane fusion activity *in vitro*, and with inhibition of catecholamine secretion *in vivo*.

We therefore conclude that the exocytotic machinery includes ANX7 as a common site of action for calcium, GTP, PKC and Botulinum toxin in the exocytotic membrane fusion process.

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by

Hung Caohuy

Dissertation submitted to the faculty of the

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in partial fulfillment of the requirement for the degree of

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For *My Wife Anh*,

My Daughter Elizabeth,

and *My Family*

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LIST OF ABBREVIATIONS

$8N_3GTP$	8-azido guanosine triphosphate
A_{540}	absorbance at 540 nm
$[AlF_4]^-$	aluminum fluoride complex
ANX1	annexin 1
ANX2	annexin 2
ANX7	annexin 7
Ba^{+2}	barium divalent cation
BoNT	botulinum toxin
BoNT/C	botulinum toxin type C
Ca^{+2}	calcium divalent cation
$CaCl_2$	calcium chloride
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
DAG	diacylglycerol
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EF-G	elongation factor G
EF-Tu	elongation factor Tu
EGTA	ethylene glycol-bis[β-aminoethylether]-N,N,N',N'-tetraacetic acid
EMEM	Eagle's minimum essential media
G_E	G protein for exocytosis

GDP	guanosine diphosphate
GDPβS	guanosine-5'-O-(2-thio-diphosphate)
GMPPNP	5'-guanylyl-imidodiphosphate
GTP	guanosine triphosphate
GTPγS	guanosine-5'-O-(3-thio-triphosphate)
HCl	hydrochloric acid
IP_3	myo-inositol (1,4,5)-trisphosphate
IPTG	isopropyl-β-D-thio-galactopyranoside
LiCl	lithium chloride
Mg-ATP	adenosine triphosphate magnesium salt
$MgCl_2$	magnesium chloride
Me_2SO_4/DMSO	dimethyl sulfoxide
MES	2-[N-morpholino] ethanosulfonic acid
$NaHCO_3$	sodium bicarbonate
$(NH_4)_2SO_4$	ammonium sulfate
NSF	N-ethylmaleimide-sensitive fusion protein
OD	optical density
P_i	inorganic phosphate
PKC	protein kinase C
PMSF	phenylmethylsulfonic fluoride
PS	phosphatidylserine
PIPES	piperazine-N,N'-bis[2-ethanesulfonic acid]
PKA_{cat}	catalytic subunit of cAMP-dependent kinase

PKG	cGMP-dependent kinase
PMA	phorbol 12-myristate 13-acetate
PVDF	polyvinylidene difluoride
SLO	streptolysin O
Sr^{+2}	strontium divalent cation
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SNARE	soluble NSF attachment protein receptor
SNAP-25	25-kDa synaptosome-associated protein
SNAPS	soluble NSF attachment proteins
TeNT	tetanus toxin
TPA	2-O-tetradecanoyl-phorbol-13-acetate
VAMP	vesicle associated membrane protein

CHAPTER 1

Introduction and Background

Exocytosis

Exocytosis involves fusion of secretory vesicle membranes with the inner surface of the plasma membrane of the cell. This process is of fundamental importance in biology as it represents the route by which many neuro-transmitters, hormones, and enzymes are secreted (Schweizer *et al*., 1991**;** Morgan, 1994). Although the regulation of exocytosis has been studied in detail for many cell types, there is little known about the underlying mechanisms involved in the exocytotic membrane fusion. One of the reasons for this is that it has been difficult to establish *in vitro* fusion systems between secretory vesicles and the plasma membrane. The availability of such a system would allow identification of the required protein(s) acting either as a single fusogen or a scaffold. The other reason is that the exocytotic process is regulated by several interacting second messenger systems.

Models Systems for Studying Exocytosis

One approach to elucidate the intracellular mechanisms underlying exocytosis in secreting cells involves permeabilization of the plasma membrane, which permits manipulation of the intracellular environment. The current permeabilization techniques used for this approach include electroporation (Knight and Baker, 1982), detergent digitonin (Dunn and Holz, 1983; Wilson an

Kirshner, 1983), and bacterial toxins (Bader *et al*., 1986). Over the years, such permeabilized cell studies have established the primary roles of calcium, guanine nucleotides, and protein kinases in regulated exocytosis.

Role of Calcium in Exocytosis

The importance of calcium in the regulation of secretion has long been recognized (Winkler, 1988). Several studies using the photoprotein aequorin and Ca^{+2}-sensitive, membrane-permeant, fluorescent dyes (i.e. quin-2, fura-2, and indo-1) have shown for a number of cell types that secretion is associated with an increase in intracellular Ca^{+2} concentration (Tsien *et al*., 1984; Rink, 1988). Unfortunately, little information could be concluded about the magnitude of intracellular Ca^{+2} concentration at the site of exocytosis. Low Ca^{+2} concentrations in the range of 1-10 μM have been shown to be sufficient to induce secretion from permeabilized cells (Burgoyne *et al*.,1991). However, a number of other studies have indicated that higher Ca^{+2} concentrations in the 100 μM range are required to trigger the final fusion step of secretion (Bittner and Holz, 1992; Hay and Martin, 1992; Howell *et al*., 1989; Gompert *et al*., 1990; Neher and Zucker, 1993; Thomas *et al*., 1993). One particular kinetic study, performed by Bittner and Holz (1992), has shown that low Ca^{+2} concentrations (10 μM or less) do indeed induce secretion, but that the initial rates of secretion under these low Ca^{+2} concentrations proceed markedly slower, as compared to those which proceed much more rapidly at higher Ca^{+2} concentrations ($\geq$ 100 μM). In support of the latter conclusion, electrophysiological studies with caged

calcium in intact and digitionin-permeabilized chromaffin cells (Augustine and Neher, 1992; Neher and Zucker 1993; von Ruden *et al*., 1993; Henemann *et al*., 1994; Bittner and Holz, 1992) and neurons (Thomas *et al*., 1993; Heldelberger *et al*., 1994) have estimated the local Ca^{+2} concentration in the region immediately subadjacent to the exocytotic site to be increased into the 50-300 μM range. These results have been further supported in recent studies on the squid stellate ganglion (Llinas and Sugimori, 1997) and retinal bipolar neurons (Heldelberger, 1997). Thus, the Ca^{+2} concentrations beneath the membrane necessary to induce exocytosis in intact cells may be in the order of 50-300 μM rather the submicromolar concentrations.

Role of Guanine Nucleotide in Exocytosis

A rise in cytosolic calcium is a common stimulus for exocytosis. However, it is not the only initiator of regulated exocytosis. Indeed, it has been demonstrated that guanine nucleotides are able to induce or enhance exocytotic secretion from many cell types by a mechanism thought to involve as yet unknown proteins in the GTPase superfamily (Neher, 1988**;** Fernandez *et al*., 1984). Experiments using cells pretreated with agents, which selectively deplete the cytosolic levels of GTP (i.e. mycophenolic acid or ribavirin), have further supported the role of GTP-binding proteins in exocytosis. Cells treated with these agents fail to undergo secretion either in response to receptor-directed ligands or to the Ca^{+2} ionophore A23187 (Marquardt *et al*., 1987; Wilson *et al*., 1988). Nearly all evidence for the involvement of GTP-binding proteins in exocytosis come from

experiments with permeabilized cells using the hydrolysis-resistant analogues of GTP (i.e. GTPγS and GMP-PNP). Results obtained from these experiments suggest that G-proteins may be involved in exocytosis at stages other than that of signal transduction. This has been found in several cell types, including platelets (Haslam *et al*., 1984), mast cells (Howell *et al*., 1987), neutrophils (Barrowman *et al*., 1986), insulinoma RINm5F cells (Vallar *et al*., 1987), AtT-20 cells (Luini and De Matteis, 1990), parathyroid cells (Oetting *et al*., 1986), and chromaffin cells (Knight and Baker, 1985; Bittner *et al*., 1986; Bader *et al*., 1989**;** Ahnert-Hilger *et al*., 1992). Support for this suggestion comes from the observation that exocytosis stimulated by Ca^{+2}-plus-GTPγS can be fully maintained under the conditions in which neomycin suppresses G-protein linked phospholipase C, which cleaves phosphatidylinositol 4,5-bisphosphate to generate two messengers, inositol (1,4,5)-trisphosphate (IP_3) and diacylglycerol (DAG) (Cockcroft *et al*., 1987). This finding thus strengthens the contention that a second GTP-binding protein, namely G_E (G-protein for exocytosis), is involved at a late stage in the stimulus-secretion process.

The available data have been interpreted to indicate that G_E acts at a site distal to the second messenger system in the exocytotic process. However, these studies do not address the issue of whether G_E represents a heterotrimeric G-protein and/or a monomeric G-protein, since both are activated by GTP analogues. With the availability of the GTP-binding “probes”, G_E has been proposed to be homologous to a class of heterotrimeric G-proteins. Specific examples to support this proposal come from the studies using mastoparan and

aluminum fluoride (AlF_4^-). In the first example, mastoparan, a tetradecapeptide toxin from wasp venom, is known to activate heterotrimeric G-proteins by promoting GDP/GTP exchange through a mechanism that is virtually identical to that of agonist-liganded receptors (Higashijima *et al*., 1988; 1990). Mastoparan (MP) has been found to stimulate secretion from a variety of cell types, including mast cells (Mousli *et al*., 1989), platelets (Wheeler-Jones *et al*., 1992), anterior pituitary cells (Kurihara *et al*., 1986), chromaffin cells (Vitale *et al*., 1993; 1994), and pancreatic islets of Langerhans (Yokokawa *et al*., 1989; Jones *et al*., 1993; Straub *et al*., 1998). Furthermore, MP-induced insulin secretion is not inhibited by down-regulation of islet protein kinase C, but is inhibited by the presence of GDPβS (Jones *et al*., 1993). In the second example, aluminum fluoride anion (AlF_4^-) has been shown to activate G-proteins at the nucleotide-binding site by forming a GDP-Al_3^+-F_3^- complex that imitates GTP (Bigay *et al*., 1987). This anion selectively activates heterotrimeric, but not small GTP-binding proteins (Kahn, 1991). Similarly to the MP effect, AlF_4^- does stimulate a number of cell types to undergo Ca^{+2}-dependent secretion. These cell types include mouse Paneth cells (Satoh *et al*., 1992), rabbit polymorphonuclear leukocytes (Elferink and Deierksauf, 1989), RINm5F β cell line (Komatsu *et al*., 1995), as well as permeabilized pancreatic acini (Padfield and Panesar, 1998), and chromaffin cells (Kuroda *et al*., 1980).

Role of Protein Phosphorylation in Exocytosis

There is considerable interest in the possibility that protein phosphorylation may also play a role in the process of stimulus-secretion process. Indeed, many studies have shown that Mg-ATP is required for secretion in chromaffin and other cell types (Baker and Knight, 1983; Dunn and Holz, 1983; Wilson and Kirshner, 1983; Knight and Scrutton, 1980). Several other studies have further found that depletion of ATP, either before or after permeabilization, causes a decrease in protein phosphorylation and consequently inhibits secretion (Nakanishi *et al.,* 1988; Morita *et al*., 1988). From these findings, it has been concluded that ATP is required in the secretory process as a substrate for protein kinases.

Considerable evidence has strongly indicated that exocytosis is modulated by protein kinase C (PKC), but not by other protein kinases, such as cAMP-dependent protein kinase (Bittner *et al*., 1986; Knight and Baker, 1982) and cGMP-dependent protein kinase (O'Sullivan and Burgoyne, 1990; Ely *et al*., 1990). Indeed, activation of PKC in bovine chromaffin cells, for example, with tumor-promoting phorbol esters (Pocotte *et al*., 1985; Brocklehurst *et al*., 1985), or with other secretagogues, i.e. nicotine and carbachol, (TerBush and Holz, 1986; TerBush *et al*., 1988), causes a significant increase in catecholamine secretion in a Ca^{+2}-dependent manner. By contrast, secretion is reduced when PKC activity is down regulated by 24-hr pretreatment with phorbol esters (Burgoyne et al., 1988), or is inhibited using various specific PKC inhibitors (Isosaki *et al*., 1994; TerBush and Holz, 1990; Tachikawa *et al*., 1990). The stimulatory effect of PKC activation on exocytosis has also been reported in

various other cell types, including platelets (Rink *et al*., 1983), neutrophils (Smolen *et al*., 1989), pituitary cells (Stojikovic *et al*., 1991), insulin-secreting β cells (Persaud *et al*., 1989), and mast cells (Howell *et al*., 1989; Koopmann and Jackson, 1990).

In addition, permeabilized cell studies have further suggested that PKC, calcium, and guanine nucleotides may exert their positive actions either directly on a common site, or on putative target proteins that are closely associated with each other in the exocytotic machinery. The reason is that activation of one element synergistically reduces the concentrations of the others required for optimal secretion. For example, activation of PKC by phorbol ester TPA (12-O-tetradecanoyl-phorbol-13-acetate) increases the Ca^{+2}-dependent secretory response obtained at submaximal concentrations of GTPγS (Cockcroft *et al.,* 1987; Ahnert-Hilger *et al*., 1987; Lillie and Gomperts, 1991), whereas depleting the PKC activity by 24 hr pretreatment with TPA suppresses the stimulatory effect of GTPγS (Bader *et al*., 1989). Similarly, PKC activators also enhance the affinity for calcium of secretion by inducing a leftward shift of the Ca^{+2}-activation curve with little effect at very low and very high concentrations of calcium (Knight and Baker, 1983; Knight *et al*., 1988).

Taken together, these studies provide substantial evidence to implicate the essential roles of calcium, guanine nucleotide, and protein kinase C in regulated exocytosis. However, the specific sites of action for these effectors in the stimulus-secretion cascade remain unknown.

Gomperts' Model for Exocytosis (version 1.0)

These results thus have led to the proposal of a hypothetical exocytotic model by Gomperts and his co-workers, as illustrated in Fig. 1 (Gomperts, 1990). This model has emphasized the importance of protein kinase C and unidentified binding proteins for calcium and GTP at the docking/fusion site. In this model, there are two GTP-binding proteins involved in controlling the stimulus-secreting process. The first G protein is the putative G_p, a receptor-linked GTP-binding protein, which induces the activation of phospholipase C. Activated phospholipase C in turn cleaves phosphatidylinositol 4,5-bisphosphate to generate two messengers, inositol (1,4,5)-triphosphate (IP_3) and diacylglycerol (DAG). Elevated IP_3 causes an increase in intracellular calcium, whereas DAG activates Ca^{+2}- and phospholipid-dependent protein kinase C. An increase in intracellular calcium leads to activation of Ca^{+2}-binding proteins, while activation of protein kinase C leads to protein phosphorylation of both Ca^{+2}-binding proteins and the second G protein (G_E), which acts further downstream from the signal transduction level. G_E refers to a hypothetical G protein specializing in exocytosis. These proteins are thought to be responsible for exocytosis. In addition, this model has further emphasized that the putative Ca^{+2} and GTP target proteins may be closely associated with each other at the fusion site, since activation of one effector reduces the concentrations of other effectors required for optimal secretion.

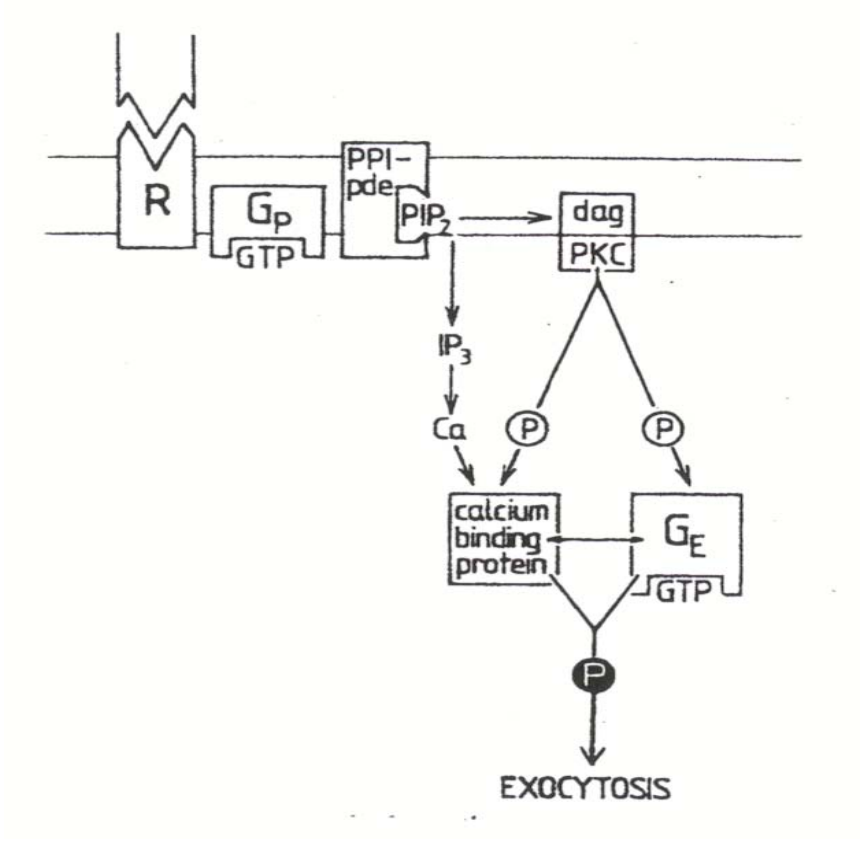

Figure 1. **A schematic representation of the relationships between the early events in stimulus-secretion coupling and late events mediated by the GTP- and calcium-binding proteins.** Two GTP-binding proteins are involved in controlling the stimulus-secretion pathway. The first GTP-binding protein is the putative receptor-linked G-protein (G_P) that controls the activity of phospholipase C, thereby generating inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). Further downstream from the signal transduction level, a second GTP-binding protein (G_E, E for exocytosis), a putative GTPase so far undefined as a molecular entity, acts in parallel, or might be closely associated with a Ca^{2+}-binding protein at the docking/fusion site of the exocytotic machinery. Subsequent phosphorylation by DAG-activated PKC triggers these proteins into mediating the exocytotic membrane fusion process. From Gomperts B. D. (1990).

Annexins in Exocytosis

Several classes of Ca^{+2}-binding proteins have been hypothesized as possible mediators for exocytosis. However, their roles in the exocytotic events have not been conclusively established. Prominent among these possible protein mediators are the annexins. Annexins are members of a large gene family that exhibit Ca^{+2}-dependent binding to phospholipid vesicles (Raynal and Pollard, 1994). Several annexins (e.g. annexins 1, 2, and 7) can cause the secretory vesicles to aggregate and fuse *in vitro*, suggesting that they might play a role in regulating membrane fusion. Indeed, annexin 1 and annexin 2 are found to be phosphorylated by protein kinase C both *in vivo* (Michener *et al*., 1986; Gould *et al*., 1986) and *in vitro* (Wang and Creutz, 1992; Johnstone *et al*., 1992; 1993). However, phosphorylation of these proteins by protein kinase C markedly inhibits their aggregation and fusion activities *in vitro* (Wang and Creutz, 1992; Johnstone *et al*., 1992; 1993), thus leading to the conclusion that they are unlikely to mediate the positive action of protein kinase C on exocytosis. The opposite is the case for annexin 7, and so serves to direct interest to annexin 7.

Annexin 7 (ANX7; synexin) is a Ca^{+2}-dependent phospholipid binding protein (Creutz *et al*., 1978; Creutz *et al*., 1979; Raynal and Pollard, 1994) that occurs predominantly in an intracellular, vesicle- and plasma membrane-bound state (Cardenas *et al*., 1994; Kuijpers *et al*., 1992). *In vitro*, ANX7 has the ability to promote chromaffin granule aggregation (Creutz *et al*., 1978) and fusion of granule membranes (Creutz *et al*., 1982; Nir *et al*., 1987; Stutzin, 1986; Stutzin *et*

al., 1987) and acidic phospholipid liposomes (Hong *et al*., 1981; Hong *et al*., 1982) in a Ca^{+2}-dependent manner. The mechanism of membrane fusion mediated by ANX7 has been hypothesized to be involved the formation of a "hydrophobic bridge" between fusion membrane partners (Pollard *et al*., 1991). From this perspective, we have proposed that the membrane fusion protein ANX7, with an intrinsic K_d for calcium of *ca.* 200 μM (Raynal and Pollard, 1994), might contribute to the hypothetical GTP/Ca^{+2}-sensitive exocytotic site. As indicated above, the sites of action for calcium and GTP have been hypothesized to be closely associated in a common pathway (see Gomperts' model for exocytosis). Indeed, we have recently reported that ANX7 is a unique Ca^{+2}-conditional GTP-binding protein, and that *in vitro* chromaffin granule aggregation and phosphatidylserine liposome fusion activities of ANX7 are substantially enhanced upon binding to GTP and deactivated by GTP hydrolysis (Fig. 2; Caohuy *et al*., 1996). Likewise, studies of intact chromaffin cells indicate that ANX7 behaves in a similar fashion *in vivo* (Fig. 3; Caohuy *et al.*, 1996). Furthermore, ANX7 behaves more like the heterotrimeric G-proteins than small monomeric G-proteins, although its GTP-binding consensus motifs are similar to those of Ras, a small monomeric G-protein (Caohuy and Pollard, unpublished data). For example, we have reported that the GTP binding and GTPase activities of ANX7 are substantially activated by mastoparan, a known heterotrimeric G-protein activator (Caohuy *et al*., 1998). Furthermore, $[AlF_4]^-$, another heterotrimeric G-protein activator, also causes a significant dose-dependent increase in the Ca^{+2}-dependent chromaffin granule aggregation and

phosphatidylserine liposome fusion activities of ANX7 (Caohuy, unpublished data).

In addition to the requirement of calcium, other divalent cations, such as Sr^{+2} and Ba^{+2}, have been shown to potently activate exocytosis in neurons and other secretory cells (TerBush and Holz, 1992; Boonen *et al*., 1993; Ruden *et al*., 1993; Barnett and Misler, 1995). Interestingly, ANX7-driven chromaffin granule aggregation (Zaks and Creutz, 1990) and GTP binding of ANX7 (Caohuy *et al*., 1996) can be activated by these divalent cations. Collectively, these findings thus have led us to give fuller credence to the possibility of the involvement of ANX7 in the exocytotic membrane fusion process. More importantly, this hypothesis is further supported by our recent report showing that a nullizygous (-/-) knockout of the Anx7 gene in mouse is lethal, while insulin secretion from islets of Langerhans of the heterozygous anx7 (+/-) mouse is found to be defective (Srivastava *et al*., 1999).

SNARE Model for Exocytosis (version 2.0)

Other possible mediators for exocytosis are protein components predicted by the SNARE hypothesis (Rothman, 1994; Rothman and Orci, 1992**;** Sudhof, 1995**;** Chapman *et al*., 1995; Burgoyne and Morgan, 1998). As shown in Fig. 4, this hypothesis envisions a core complex formed between two plasma membrane proteins [syntaxin and 25-kDa synaptosomal-associated protein (SNAP-25)] and two synaptic vesicle proteins [synaptobrevin/ VAMP and

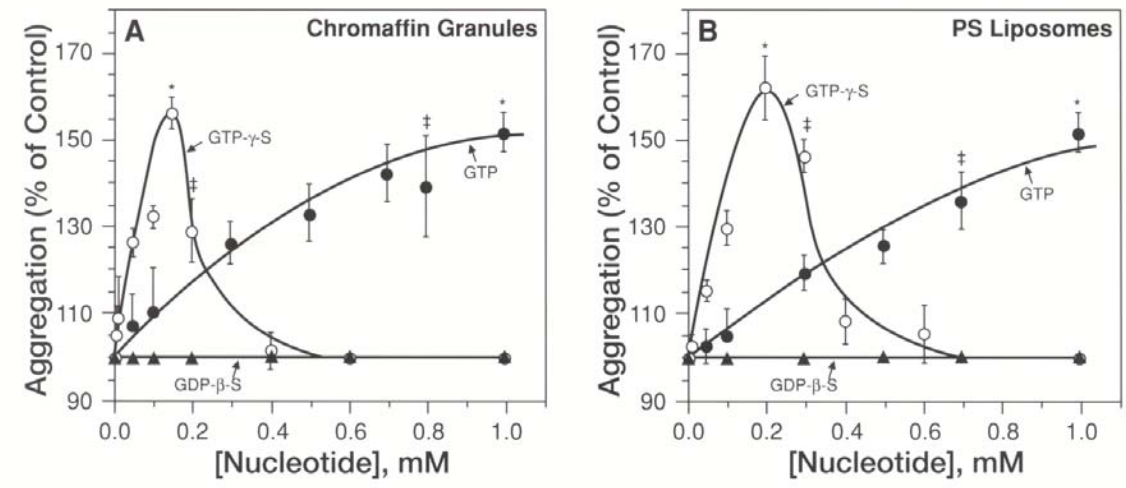

Figure 2. **Effect of GTP and GTP analogues on ANX7 activity.** (***A***) ANX7-driven aggregation of chromaffin granules. (***B***) fusion of PS liposomes. Various concentrations of GTP (●), GTPγS (O), or GDPβS(▲) were added, and the aggregation and fusion reactions were measured by the change in absorbance at 540 nm for 30 min. From Caohuy *et al*. (1996).

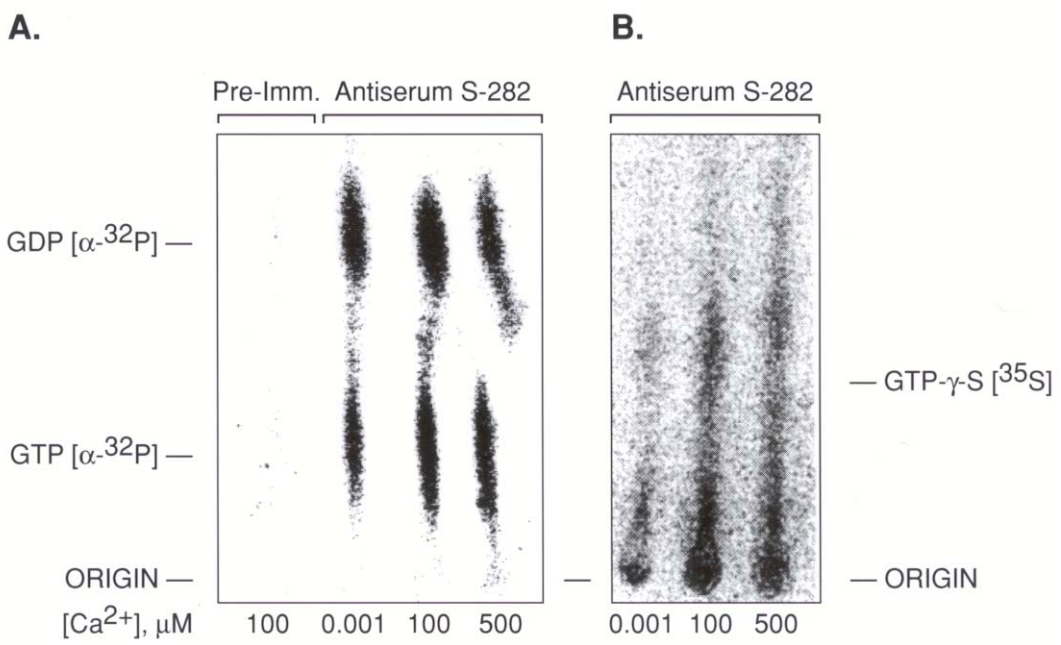

Figure 3. ***In vivo* labeling of ANX7 by GTP in SLO-permeabilized chromaffin cells.** Thin layer chromatogram of the nucleotides eluted from immuno-precipitates of ANX7 from SLO-permeabilized chromaffin cells that had been incubated in different concentration of Ca^{+2} with either [α-^{32}P]GTP (***A***) or GTP[γ-^{35}S] (***B***). Migration positions of guanine nucleotide standards are shown at the left or right, respectively. From Caohuy *et al*. (1996).

synaptotagmin]. This interbilayer complex formation subsequently promotes vesicle targeting and docking to the plasma membrane. Thereafter, the assembled SNARE complex then acts as a receptor for the SNAPS [soluble NSF attachment proteins], which in turn binds the fusion protein, NSF [N-ethylmaleimide-sensitive fusion protein] to the complex. NSF, an ATPase, then catalyzes the ATP-dependent disassembly of the SNARE complex. These data have led to the hypothesis that this disruption is an intrinsic, essential event that initiates membrane fusion in the exocytotic pathway (Sollner, 1995; Rothman and Sollner, 1997).

However, the salient fact is that none of these SNARE protein components can actually fuse membranes. NSF, which has been shown to play an important role in fusion of Golgi-derived transport vesicles (Malhotra *et al*., 1988), appears not to be involved in fusion itself, but rather in the preparation for fusion. In addition, NSF and ATP are not needed for the final steps in Ca^{+2}-triggered exocytosis in permeabilized neuroendocrine cells (Bittner and Holz, 1992; Banerjee *et al*.,1996). Recently, Weber and his colleagues (1998) have shown that the formation of a SNARE complex can drive an extraordinarily slow liposome fusion process *in vitro*. Indeed, the time course for this SNARE-driven fusion process is measured in hours. This process is thus far too slow to account for fast milisecond exocytosis. By contrast, annexin 7 can fuse liposomes *in vitro* with impressively faster kinetics ($\approx$ 4 μsec; Raynal and Pollard, 1994), and is essentially diffusion-limited.

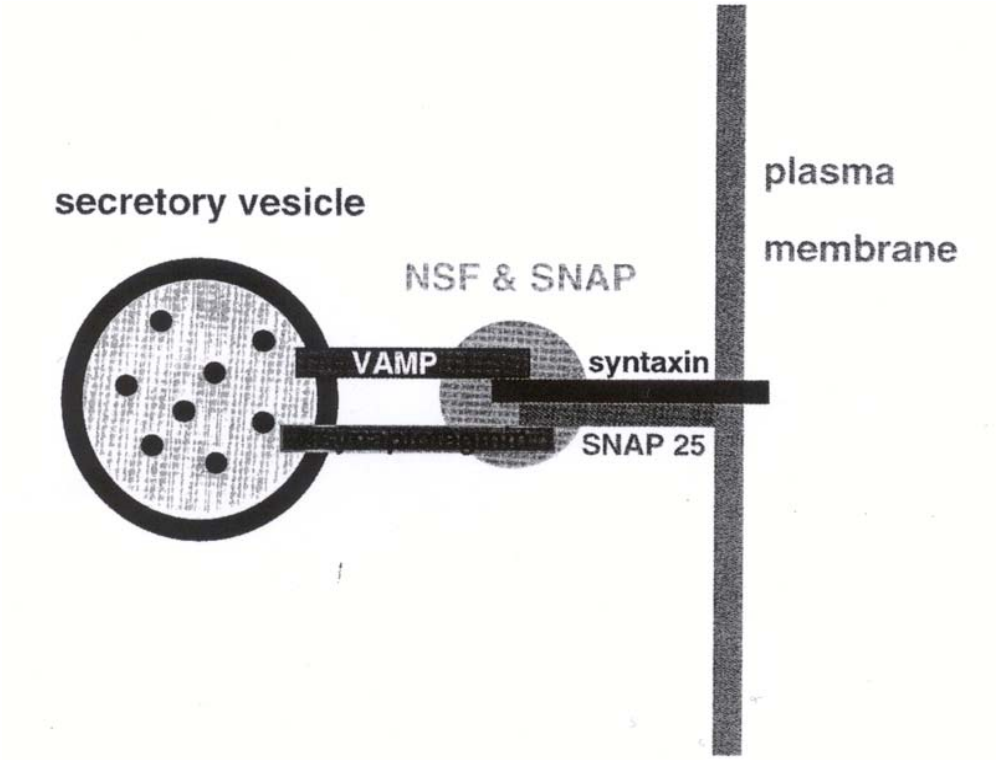

Figure 4. **The SNARE hypothesis of exocytosis**. A core complex formed between two plasma membrane proteins [syntaxin and 25-kDa synaptosomal-associated protein (SNAP-25)] and two synaptic vesicle proteins [synaptobrevin/ VAMP and synaptotagmin]. This interbilayer complex formation is mediated by NSF and SNAPs. From Rothman J. E. (1994).

Moreover, recent works on the sea urchin egg cortical vesicle fusion system (Coorssen *et al*., 1998; Tahara *et al*., 1998) have argued against the role of the SNARE complex in the Ca^{+2}-triggered membrane fusion process. These isolated cortical vesicles have shown to retain Ca^{+2} sensitivity for fusion, carrying with them all the molecular machinery necessary for docking, Ca^{+2} sensing, and membrane-membrane fusion (Vogel and Zimmerberg, 1992). Interestingly, these studies have found that calcium can induce isolated cortical vesicle fusion either in the absence of SNARE complex disruption, or under conditions causing the complete loss of complexes. In addition, under conditions of complete SNARE complex disruption, cortical vesicles still remain firmly attached to the plasma membrane and retain full competence for Ca^{+2}-triggered fusion. These findings thus have led to the suggestion that other unidentified docking/fusion components may have existed in the docking/fusion machinery. This suggestion is further supported by the data showing that neither Sr^{+2} nor Ba^{+2} can cause SNARE complex disruption although they support near-maximal fusion (Coorssen *et al*., 1998). These data are comparable to data on Sr^{+2}- and Ba^{+2}-triggered exocytosis in secretory cells (TerBush and Holz, 1992; Boonen *et al*., 1993; Ruden *et al*., 1993; Barnett and Misler, 1995). In conclusion, these findings tend to exclude the role of the SNARE complex in initiating membrane fusion. However, they do not rule out a contribution from these proteins to the efficiency of Ca^{+2}-triggered fusion. For example, when SNARE complexes are not allowed to form, cortical vesicles still dock to one another sufficiently well and

undergo Ca^{+2}-triggered fusion, albeit at Ca^{+2} concentrations higher than required in the presence of SNARE complexes (Coorssen *et al*., 1998).

Because of the ability of synaptotagmin to bind to phospholipids (Brose *et al*., 1992) and to syntaxin (Li *et al*., 1995) in a Ca^{+2}-dependent manner, this protein has been proposed to act as a Ca^{+2} sensor for mediating the membrane fusion process (DeBello *et al*., 1993). However, disruption of the synaptotagmin gene in mice produces animals that are virtually normal in all ways, except for a loss in the fast component of Ca^{+2}-dependent exocytosis (Broadie *et al*., 1994; Geppert *et al*., 1994). These results thus tend to exclude synaptotagmin from direct and essential involvement in the fusion step. Yet they do not exclude the possibility that synaptotagmin is either a calcium sensor immediately prior to docking, or important in some other way for the efficiency of the Ca^{+2}-dependent secretory process.

In addition, several members of the rab family of small GTP-binding proteins have been identified in mammalian cells and yeast as having a function in vesicle trafficking (Oberhauser *et al*., 1992). These proteins are also found to be associated with the SNARE fusion machine (White, 1992), and they have therefore been hypothesized to mediate the stimulatory effect of GTP on exocytosis. Nonetheless, while they may be important for targeting and docking, they do not appear to participate in the membrane fusion step. This hypothesis is supported by recent evidence showing that in Rab3A-deficient mice the size of the readily release pool of vesicles is normal, and exocytotic events occur with ever greater frequency than in control mice (Geppert *et al*., 1997).

The interaction of SNAP-25 with syntaxin has been reported to promote the subsequent interaction between syntaxin and synaptobrevin/VAMP. This suggests that SNAP-25 might regulate the formation of the SNARE complex (Hayashi *et al*., 1994). However, it has recently been reported that phosphorylation of SNAP-25 by protein kinase C actually decreases the interaction between syntaxin and SNAP-25. Thus, protein kinase C makes the formation of the SNARE complex less likely. These data suggest that the positive action of protein kinase C on exocytosis is thus not likely to be mediated by SNARE proteins (Shimazaki *et al*., 1996).

Clostridial Neurotoxins as Specific Inhibitors of Exocytosis

Tetanus and botulinum neurotoxins are the most potent toxins known (mouse lethal dose <0.1 ng/kg), and are entirely responsible for the diseases of tetanus and botulism, respectively. Botulinum neurotoxin (BoNT) penetrates motor neurons at the neuromuscular junction and blocks acetylcholine release, thus causing flaccid paralysis. In contrast, tetanus neurotoxin (TeNT), which is also taken up into nerve endings at the neuromuscular junction, is transported in a vesicular compartment with motor axons to the soma of motor neurons within the spinal cord. At the soma of motor neurons, TeNT is transcytosed out and into adjacent synaptic terminals. TeNT appears to act preferentially on inhibitory synapses, causing motor disinhibition, leading to spastic paralysis (Simpson, 1981; Montecucco and Schiavo, 1993; 1994).

Investigations into the mechanism of the secretory process have been greatly aided by the use of these specific neurotoxin inhibitors of exocytosis (Dolly *et al*., 1994). BoNT and TeNT are produced by members of the *Clostridia botulinum* family (types A-G) and the closely related *Clostridium tetani*, respectively. These proteins consist of a heavy chain (H; 100 kDa) and a light chain (L; 50 kDa), linked together by disulfide and noncovalent bonds. Whereas the heavy chain is responsible for cell binding and subsequently internalization, the light chain alone acts intracellularly to block neurotransmitter release (Simpson, 1981; Montecucco and Schiavo, 1993; 1994). Many studies have shown that the light chains of these clostridial neurotoxins are zinc-dependent proteases, and that the proteolytic activities of these toxins are directed against three specific synaptic proteins that have been proposed to be essential in synaptic vesicle exocytosis (Niemann *et al*., 1994; Schiavo *et al*., 1992a). BoNT/B, /D, /F, /G, and TeNT recognize and cleave the vesicle-associated membrane protein, VAMP/synaptobrevin (Schiavo *et al*., 1992b**;** 1993a**;** 1994; Yamasaki *et al*., 1994a-b; Pellizzari *et al*., 1996). BoNT/A and BoNT/E recognize and cleave a synaptosome-associated membrane protein of 25-kDa, SNAP-25 (Schiavo *et al*., 1993b; Blasi *et al*., 1993b; Binz *et al*., 1994). BoNT/C cleaves syntaxin and SNAP-25 (Blasi *et al*., 1993a; Schiavo *et al*., 1995). Furthermore, a number of studies have shown that clostridial neurotoxins also block exocytosis from adrenal chromaffin cells (Knight, 1986; Lawrence *et al*., 1996; Marxen *et al*., 1991; Foran *et al*., 1996; Glenn and Burgoyne, 1996), and from insulin-secreting β-cell lines, HIT-15 and RINm5F (Regazzi *et al*., 1995; Sadoul *et al*., 1995; Boyd

et al., 1995). As described in synaptic nerve terminals, a similar set of SNARE proteins, synaptobrevin, SNAP-25, and syntaxin, are found to be cleaved by these neurotoxins as well, leading to the inhibition of secretion from these neuroendocrine cells.

Although the identification of syntaxin, SNAP-25, and synaptobrevin as targets for the clostridial neurotoxins demonstrates a function in exocytosis, the mechanism by which these proteins contribute to exocytotic membrane fusion remains enigmatic. Nonetheless, the fact that these neurotoxins hydrolyze SNARE proteins has been taken as prima facie evidence for the SNARE hypothesis for exocytotic membrane fusion. As shown in later parts of this thesis, BoNT/C binds ANX7 and cleaves it, both *in vitro* and *in vivo*.

Significance

Understanding the exocytotic process is of profound importance for understanding a spectrum of phenomena, ranging from unicellular defense mechanisms to endocrine regulatory functions, neurotransmission, and immune cell action. Over the past several decades, considerable progress has been made in understanding the organization of the secretory pathway and the molecular basis of vesicular transport. However, until now, the regulation of the final step of the secretory pathway, membrane fusion, remains unresolved. The aim of this research project has therefore been to determine the molecular basis of the membrane fusion process of regulated exocytosis, with a specific emphasis on the role of annexin 7 in this process. Our working hypothesis is that

the membrane fusion machinery might include annexin 7 as a common site of action for calcium, GTP, PKC and BoNTs in the exocytotic membrane fusion process. The experimental strategies designed to test this hypothesis include the reconstituted membrane fusion system using artificial liposomes and isolated adrenal chromaffin cells, together with other specific, sensitive functional assays *in vitro*. It is also hoped that these fundamental studies on the mechanism of exocytosis may also provide a basis for understanding the action of drugs that affect hormone or neurotransmitter release, and also lead to the development of new pharmacological agents that influence this fundamental process.

CHAPTER 2

Activation of Annexin 7 by Protein Kinase C *in vitro* and *in vivo*

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Abstract

Annexin 7, a Ca^{+2}/GTP-activated membrane fusion protein, is preferentially phosphorylated in intact chromaffin cells, and the levels of annexin 7 phosphorylation increase quantitatively in proportion to the extent of catecholamine secretion. Consistently, various protein kinase C inhibitors proportionately reduce both secretion and phosphorylation of annexin 7 in these cells. *In vitro*, annexin 7 is quantitatively phosphorylated by protein kinase C to a mole ratio of 2.0, and phosphorylation is extraordinarily sensitive to variables such as pH, calcium, phospholipid, phorbol ester and annexin 7 concentration. Phosphorylation of annexin 7 by protein kinase C significantly potentiates the ability of the protein to fuse phospholipid vesicles and lowers the half-maximal concentration of calcium needed for this fusion process. Furthermore, other protein kinases, including cAMP-dependent protein kinase, cGMP-dependent protein kinase, and protein tyrosine kinase $pp60^{c\text{-}src}$, also label annexin 7 with high efficiency, but do not have this effect on membrane fusion. In the case of $pp60^{c\text{-}src}$, we note that this kinase, if anything, modestly suppresses the membrane fusion activity of annexin 7. These results thus lead us to hypothesize that annexin 7 may be a positive mediator for protein kinase C action in the exocytotic membrane fusion reaction in chromaffin cells.

Introduction

Protein kinase C (PKC) and possibly other protein kinase activators are believed to play a regulatory role in exocytotic secretion of hormones and neurotransmitters. Indeed, activation of PKC in bovine chromaffin cells, for example, with tumor-promoting phorbol esters (Pocotte *et al.*, 1985; Brocklehurst *et al*., 1985), or other secretagogues (TerBush and Holz, 1986; TerBush *et al.*, 1988), causes an increase in catecholamine secretion in a Ca^{+2}-dependent manner. By contrast, secretion is reduced when PKC activity is down regulated by 24 hr pre-treatment with phorbol esters (Burgoyne *et al*., 1988), or is inhibited using various PKC inhibitors (Isosaki *et al*., 1994; TerBush and Holz, 1990; Tachikawa *et al*., 1990). The stimulatory effect of PKC activation on exocytosis has also been reported in various other cell types, including platelets (Coorsen *et al*., 1990), neutrophils (Smolen *et al*., 1989), pituitary cells (Stojikovic *et al*., 1991), insulin-secreting cells (Persaud *et al*., 1989; Wollheim and Regazzi, 1990), and mast cells (Howell *et al*., 1990; Churcher and Gomperts, 1990). Although phenomenologically well known, the specific sites of action of PKC in the stimulus-secretion cascade remain unknown.

The SNARE hypothesis has been proposed to explain the interactions between vesicle and plasma membranes during the period preceding exocytosis (Rothman, 1994). In this model, a Ca^{+2}-independent core complex is formed between plasma membrane protein syntaxin and SNAP-25 and the synaptic vesicle protein synaptobrevin/VAMP. Vesicular synaptotagmin is identified as a low-affinity Ca^{+2} sensor for subsequent exocytosis (Chapman *et al*., 1995). Additional evidence

suggests that *trans*-SNARE pairing may precede membrane fusion but is not be required during fusion (Ungermann *et al*., 1998; Peters and Mayer, 1998; Coorsen *et al*., 1998; Tahara *et al*., 1998). In addition, the preceding interaction of SNAP-25 with syntaxin is found to enhance the interaction between syntaxin and synaptobrevin/VAMP, suggesting that SNAP-25 regulates the formation of the SNARE complex (Hayashi *et al*., 1994). However, it has recently been reported that the phosphorylation of SNAP-25 by PKC actually decreases the interaction between syntaxin and SNAP-25. Thus, PKC makes the formation of the SNARE complex less likely. These data therefore suggest that the positive action of PKC on exocytosis is not likely to be mediated by SNARE proteins (Shimazaki *et al*., 1996).

Alternatively, annexins have also been considered as possible mediators of exocytosis. Annexin 1 (ANX1), annexin 2 (ANX2) and annexin 7 (ANX7), which are members of the annexin family, have the ability to aggregate and fuse lipid vesicles (Raynal and Pollard, 1994). Such a result has been interpreted to suggest that they might play a role in regulating membrane fusion. Indeed, both ANX1 and ANX2 are found to be phosphorylated by PKC both *in vivo* (Michener *et al*., 1986; Gould *et al*., 1986) and *in vitro* (Wang and Creutz, 1992; Johnstone *et al*., 1993; 1992). However, phosphorylation of these proteins by PKC markedly inhibits their aggregation and fusion activities *in vitro* (Wang and Creutz, 1992; Johnstone *et al*., 1993; 1992), indicating that they are also unlikely to mediate the positive action of PKC on exocytosis.

Annexin 7 (ANX7; synexin), which fuses membranes in a Ca^{+2}-dependent manner (Creutz *et al*., 1982; Hong *et al*., 1982; Nir *et al*., 1987; Pollard *et al*., 1992),

has properties that have induced us to give fuller credence to the possibility of its involvement in exocytosis. We have recently reported that ANX7 is a Ca^{+2}-activated GTPase, both *in vitro* and *in vivo*, and that its GTPase activity is increased in secreting chromaffin cells (Caohuy *et al*., 1996). More recently, we have reported that the heterozygous knockout anx7 (+/-) mouse suffers from an insulin secretion deficit from islets of Langerhans, as well as defective Ca^{+2} signaling processes in β-cells (Srivastava *et al*., 1999). In addition, a homology analysis of anx7 has suggested to us the likelihood that this protein might be target for PKC (Hauptmann *et al*., 1989), and therefore a candidate for mediation of PKC action during exocytosis.

In this study, we report that ANX7 is phosphorylated in stimulated bovine chromaffin cells, and the level of ANX 7 phosphorylation is well correlated with the release of catecholamines. ANX7 is also phosphorylated *in vitro* by various kinases, including PKC, cAMP-dependent protein kinase (PKA), cGMP-dependent protein kinase (PKG), and protein tyrosine kinase $pp60^{c\text{-}src}$. Significantly, only PKC-dependent phosphorylation of ANX7 enhances the membrane fusion activity of the protein, whereas phosphorylation by other kinases do not affect this activity, or may even decrease it, as in the case of $pp60^{c\text{-}src}$. Thus, the selective activation by PKC on exocytosis *in vivo*, and the activation of ANX7 membrane fusion *in vitro*, suggests that ANX7 may act as a positive mediator of PKC for the exocytotic membrane fusion reaction in chromaffin cells.

Experimental Procedures

Isolation and Culture of Chromaffin Cells—Chromaffin cells were isolated from bovine adrenal glands by collagenase digestion and purified on a Percoll gradient as described previously (Brocklehurst and Pollard, 1990). Isolated cells were further purified by a selective plating method (Yanagihara *et al*., 1979) and maintained in a CO_2 incubator under 5% CO_2/95% air.

[^{33}P] Orthophosphoric Acid Labeling and Treatment of Chromaffin Cells with Secretagogues, PKC Activator and Inhibitors—Cultured chromaffin cells ($5x10^6$/dish, Falcon, 35 mm) were labeled with [^{33}P] P_i (0.1 mCi/ml; Amersham) in phosphate-free Eagle's MEM medium containing 10% dialyzed fetal calf serum for 8 hr at 37^0C (Tsutsui *et al*., 1994). Then, the cells were washed once with Ca^{+2}-free extracellular buffer A (buffer B without 2.2 mM $CaCl_2$ added). The cells were stimulated with extracellular buffer B [118 mM NaCl, 4.2 mM KCl, 10 mM $NaHCO_3$, 10 mM glucose, 25 mM Hepes (pH 7.2), 0.1% bovine serum albumin, 1.2 mM $MgCl_2$, and 2.2 mM $CaCl_2$] containing 100 nM phorbol 12-myristate 13-acetate (PMA; ICN), 100 μM carbachol (Sigma) or 10 μM nicotine (Sigma) for 30 min at 37^0C. After incubation, the cells were rapidly washed twice with buffer A and then solubilized in lysis buffer for immunoprecipitation. For experiments with PKC inhibitors, ^{33}P-labeled cells were preincubated for 60 min at 37^0C with or without staurosporine (50, 100, and 200 nM; Calbiochem), calphostine C (50 and 500 nM; Calbiochem) or chelerythrine (0.7 and 1.0 μM; Calbiochem) in buffer A. The cells were then stimulated for 30 min with 100 nM PMA, 100 μM carbachol or 10 μM

nicotine, followed by cell lysis for immunoprecipitation. Control experiments were performed using cells incubated with buffer B or buffer B containing DMSO (for experiments using DMSO-soluble compounds, e.g., PMA, staurosporine, calphostine C, and chelerythrine). For experiments in determining catecholamine secretion under the above conditions, parallel experiments were carried out using unlabeled chromaffin cells, and the media were then collected for measuring catecholamine concentrations.

Immunoprecipitation of ^{33}P-labeled ANX7—The cells were lysed in 1 ml of ice-cold lysis buffer [150 mM NaCl, 1% (v/v) Nonidet P-40, 0.5% (w/v) sodium deoxycholate, 5 mM EGTA, 0.2 mM Na_3VO_4, 1 mM β-glycerolphosphate, 1 μg/ml leupeptin, 1 mM PMSF, and 50 mM Tris-HCl (pH 7.5)], followed by incubation for 20 min on ice. The lysates were clarified by centrifugation at 12000 x g for 15 min at 4^0C, and the resulting lysates were precleared by incubation for 30 min with 50 μl of a 10% (v/v) suspension of protein G-Sepharose (Zymed), followed by centrifugation. The final lysates, with equal protein amounts determined by a BCA kit (Pierce), were then incubated with 10 μg of anti-ANX7 monoclonal antibody (mAb) 10E7 for 6 hr at 4^0C. Immunoprecipitates were collected on protein G-Sepharose, washed four times by pelleting in cold lysis buffer, separated by SDS-PAGE, and analyzed by PhosphorImager (Molecular Dynamics) or autoradiography.

Measurement of Catecholamine Release—The assay for catecholamine release from chromaffin cells was performed exactly as described previously

(Brocklehurst and Pollard, 1990). The release of catecholamines was expressed as a percentage of total cellular catecholamines.

Preparation of Phosphatidylserine Lipid Vesicles—PS lipid vesicles were prepared fresh daily by the swelling method (Reeves and Dowben, 1968). Briefly, highly purified (> 99%) brain phosphatidylserine (Avanti Polar Lipids) in a 1:4 chloroform-methanol solution was dried slowly under nitrogen and then allowed to swell in 0.3 M sucrose at room temperature. The suspension was then sonicated and centrifuged at 12,000 x g. The PS lipid vesicle pellet was resuspended in 0.3 M sucrose solution.

Isolation and Purification of Human Recombinant ANX7—Human recombinant ANX7 was isolated and purified as described previously (Burns *et al*., 1990). Briefly, *E. coli* bacteria containing the anx7-expressing vector (pTrc-FLS) were grown in 1 L of Luria Broth at 37^0C. After reaching an OD_{540} of 0.6, the culture was incubated overnight in the presence of 1 mM IPTG (ICN). After incubation, the bacteria were harvested by centrifugation. Expressed recombinant ANX7 was then extracted from the *E. coli* paste, concentrated by precipitation with 0-20% (w/v) $(NH_4)_2SO_4$ and purified by gel filtration using Ultragel AcA54 (Biosphere). Partially purified ANX7 was further purified by binding to PS lipid vesicles in the presence of Ca^{+2} and extracting with EGTA. This purification step was repeated six times to finally yield a highly purified ANX7 preparation ($\geq$ 98%), determined by SDS-PAGE and silver staining.

In Vitro Phosphorylation of ANX7—All phosphorylation assays using purified rat brain PKC were performed at 30^0C in a final volume of 30 μl as described elsewhere (Uchida and Filburn, 1994). Rat brain PKC with a purity of ≥95% was purchased from Calbiochem and contained the following PKC isoforms: α, β, and γ. Phosphorylation of ANX7 by PKC was examined at different pH values, and the reaction mixture contained the following: 25 mM Tris-HCl (pH 7.5), 25 mM PIPES (pH 6.8) or 25 mM MES (pH 6.1), 10 mM $MgCl_2$, 1 mM $CaCl_2$, 100 nM PMA, 400 μg/ml PS liposomes, 0.05 unit (0.035 μg) PKC, and 0.25 μg purified human recombinant ANX7. All reactions were then incubated for 30 min. In other assays to determine the conditions of ANX7 phosphorylation, 0.25 μg of ANX7 was incubated for 60 min with or without 0.05 unit of PKC in 25 mM PIPES (pH 6.8), 10 mM $MgCl_2$, and the following conditions: no lipid or Ca^{+2} added; 1 mM Ca^{+2} added, without lipid; lipid added, without Ca^{+2}; or both Ca^{+2} and lipid added. These conditions were also examined in the presence of 100 nM PMA. To determine the mole ratio of ANX7 phosphorylation by PKC, ANX7 (0.25 μg) was incubated for the indicated time periods with 0.05 unit of PKC under the optimal phosphorylation condition [25 mM PIPES (pH 6.8), 10 mM $MgCl_2$, 1 mM $CaCl_2$, 100 nM PMA, and 400 μg/ml PS liposomes]. In the assay to determine the optimal ANX7 concentration for phosphorylation, ANX7 (0.05, 0.1, 0.25, 0.5, and 1 μg) was incubated for 60 min with 0.05 unit of PKC under the optimal phosphorylation condition. The reactions containing 1 μg ANX7 were further incubated for 90, 120, and 160 min. In the assay to determine ANX7 phosphorylation as a function of Ca^{+2}

concentratrion, ANX7 (0.25 μg) was incubated for 30 min with 0.05 unit of PKC in 25 mM MES (pH 6.1) or 25 mM PIPES (pH 6.8), 10 mM $MgCl_2$, 100 nM PMA, 400 μg/ml PS liposomes, and the different free Ca^{+2} concentrations (0.01, 0.05, 0.1, 0.2, 0.3, 0.5, 0.7, and 1 mM). Free Ca^{+2} concentrations were determined as described (Fabiato and Fabiato, 1984). The above reactions were initiated by the addition of 2 μCi of [γ-^{33}P]ATP in a final concentration of 0.1 mM (3000-4000 cpm/pmol; Amersham) and stopped by the addition of the SDS-PAGE sample buffer. The phosphorylation products were analyzed by SDS-PAGE and phosphorimager analysis or autoradiography.

As for ANX7 phosphorylation by PKA, PKG, and $pp60^{c\text{-}src}$, the assays were carried out at 30^0C in a final volume of 30 μl as described (Hubaishy *et al*., 1995; Trautwein *et al*., 1994; Komalavilas and Lincoln, 1994). Purified human recombinant ANX7 (0.25 μg) was incubated for 30 min with 10 units of $pp60^{c\text{-}src}$ (Calbiochem; Hubaishy *et al*., 1995), 50 units of catalytic subunit of PKA (Promega; Trautwein *et al*., 1994) or 200 units of PKG (Promega; Komalavilas and Lincoln, 1994) plus 10 μM cGMP in 25 mM MES (pH 6.1), 10 mM $MgCl_2$, and 1 mM $CaCl_2$. All phosphorylation reactions were initiated and analyzed as described above for the PKC reactions.

Phosphoamino Acid Analysis—The phosphoamino acid analysis was performed as described (Hunter and Sefton, 1980). After autoradiography, labeled ANX7 bands were excised from the SDS-PAGE gel and then electroeluted as described by Manufacturer's instructions (BioRad). The eluate was dialyzed

overnight in water to remove SDS. The dialysate was concentrated by lyophilization and was then resuspended in 6 N HCl, followed by incubation at 110^0C for 2 hr. O-phosphoserine, O-phosphothreonine, and O-phosphotyrosine (Sigma) at 5 mM each were added to the sample, and a 5-μl aliquot was spotted on a thin layer cellulose plate (Merck), followed by electrophoresis at 1 kvolt for 30 min in a pH 3.5 buffer [pyridine/acetic acid/water, 1:33:40 (v/v)]. Unlabeled phosphoamino acids were stained with ninhydrin (0.2% in acetone), and labeled phosphoamino acids were detected using the phosphorimager.

Extraction of Phosphorylated and Unphosphorylated ANX7—Extraction of the phosphorylated and unphosphorylated protein from lipid vesicles was performed as described (Johnstone *et al*., 1992). In each of 30 reactions, 1 μg of ANX7 was incubated for 2 hr at 30^0C with 0.05 unit of PKC in 25 mM PIPES (pH 6.8), 10 mM $MgCl_2$, 1 mM $CaCl_2$, 100 nM PMA, 400 μg/ml PS liposomes, and 0.1 mM ATP in a final volume of 30 μl. Unphosphorylated ANX7 was treated in a similar manner as described for phosphorylated ANX7, except ATP was omitted. All reactions were pooled and centrifuged at 100,000 x g for 10 min, and the pellet, containing lipid and lipid-associated ANX7, was resuspended in 25 mM Tris-HCl (pH 7.5), 20 mM EGTA, and 20 mM EDTA. The mixture was sonicated in a bath type sonicator and then incubated for 30 min on ice, followed by centrifugation at 100,000 x g. The supernatant, containing ANX7, was collected and recentrifuged twice to remove residual lipid vesicles. After removing EGTA and EDTA, the concentration of phosphorylated and unphosphorylated ANX7 was determined by immunoblotting

using [^{125}I]-labeled secondary antibody and a known amount of ANX7 as a standard, and quantitated with the phosphorimager. A parallel experiment using [γ-^{33}P]ATP was also carried out to determine the stoichiometry of phosphorylation.

Lipid Vesicle Fusion Mediated by ANX7—The PS lipid vesicle fusion assay was performed as previously described (Brocklehurst and Pollard, 1990). Lipid vesicles were first diluted to an OD_{540} of 0.6 in fusion reaction buffer [0.3 M sucrose, 40 mM histidine (pH 6.1), 0.5 mM $MgCl_2$, and 0.1 mM EGTA]. Phosphorylated or unphosphorylated ANX7 (0.5 μg) was incubated with 0.5 ml of lipid vesicle suspension in a final volume of 1 ml of fusion reaction buffer. Fusion was initiated by the addition of 1 mM $[Ca^{+2}]_{final}$ and then measured by the change in the turbidity at absorbance of 540 nm (A_{540}) using a recording Hewlett-Packard spectrophotometer for 20 min at room temperature. For the Ca^{+2}-dependent lipid vesicle fusion reaction mediated by phosphorylated or unphosphorylated ANX7, similar reactions were carried out as described above. Fusion was initiated by the addition of the indicated final Ca^{+2} concentrations (0.01, 0.05, 0.4, and 1 mM) and then monitored spectrophotometrically for 20 min. Free Ca^{+2} concentrations were determined as described elsewhere (Fabiato and Fabiato, 1979) and verified using a Ca^{+2}-selective electrode.

Phosphorylation and Fusion Reaction—Simultaneous phosphorylation and lipid vesicle fusion reactions were carried out as described elsewhere (Johnstone *et al*., 1992). The reaction in a final volume of 1 ml contained 1 μg ANX7, 0.5 unit PKC, 0.3 M sucrose, 40 mM histidine (pH 6.1), 2 mM $MgCl_2$, 100 nM PMA, 100 μM

ATP, and 0.5 ml of lipid vesicle suspension. Controls were carried out in the absence of added ATP. Fusion and phosphorylation were simultaneously initiated by the addition of 1 mM $[Ca^{+2}]_{final}$ at room temperature. Fusion was measured for 30 min as described above. To confirm that ANX7 phosphorylation occurred during fusion, parallel experiments were carried out in the presence of [γ-^{33}P]ATP, and the stoichiometry was measured as described above.

Fusion and phosphorylation reactions in the presence of other kinases were carried out as described for PKC experiments above, except no PMA was added and PKC was replaced by 2000 units PKG (plus 10 μM cGMP), 500 units PKA_{cat} or 100 units $pp60^{c\text{-}src}$.

Statistical Analysis—Data are presented as means $\pm$ standard deviation (S.D.). A relationship between ANX7 phosphorylation and catecholamine secretion was assessed by a linear regression analysis (Y axis, mean value of ANX7 phosphorylation induced by PMA, carbachol or nicotine, and inhibited by various PKC inhibitors; X axis, mean value of catecholamine secretion under similar conditions as in phosphorylation). The statistical significant values (*p*) were determined by Student's t test.

Results

In Vivo Phosphorylation of ANX7 and Stimulation of Catecholamine Release from Chromaffin Cells—Using intact bovine adrenal chromaffin cells, we investigated whether ANX7 is phosphorylated under a variety of pro-secretory conditions, including treatment with PMA, carbachol, and nicotine. In these

experiments, ^{33}P-labeled cells were stimulated for 30 min with 100 nM PMA, 100 μM carbachol, 10 μM nicotine or extracellular buffer B (control), and labeled endogenous ANX7 was immunoprecipitated with mAb 10E7, followed by SDS-PAGE and PhosphorImager analysis. As shown in Fig. 1 (*AI* and *BI*), stimulation of cells with buffer B (control) results in a small amount of ^{33}P incorporation into ANX7. In contrast, labeling of ANX7 is markedly increased by about 3- to 5-fold for all agonists tested (Fig. 1, *AI* (*bar 2*) and *BI* (*bars 2* and *5*)).

As a further test for the involvement of PKC in the phosphorylation process, we examined whether the *in vivo* phosphorylation of ANX7 could be inhibited by various PKC inhibitors prior to stimulation with PMA, or with other secretagogues. For these experiments we chose not only the relatively non-selective staurosporine (Tamaoki *et al*., 1986), but also the more selective calphostine C (Kobayashi *et al*., 1989) and chelerythrine (Herbert *et al*., 1990). As shown in Fig. 1*AI*, all three inhibitors substantially reduce labeling of immunoprecipitated ANX7 from cells stimulated with 100 nM PMA. Staurosporine, at concentrations of 50, 100 and 200 nM, causes 35 ± 5, 54 ± 3 and 64 ± 7% inhibition of ANX7 labeling (mean ± S.D., n=3), respectively (*bars 3-5*). In addition, calphostine C, at concentrations of 50 and 500 nM, causes 48 ± 7 and 64 ± 3% inhibition of ANX7 labeling, respectively (*bars 6* and *7*), while chelerythrine, at concentrations of 0.7 and 1 μM, also causes 42 ± 11 and 58 ± 16% inhibition of ANX7 labeling, respectively (*bars 8* and *9*). Furthermore, calphostine C and chelerythrine both also cause a substantial reduction in labeling of immunoprecipitated ANX7 from cells stimulated with carbachol or nicotine (Fig. 1*BI*). In these experiments, 50

nM calphostine C and 0.7 μM chelerythrine cause 44 ± 2 and 45 ± 7% inhibition in carbachol-induced labeling of ANX7, respectively (*bars 3* and *4*), and these two inhibitors at the same concentrations also cause 46 ± 6 and 49 ± 4% inhibition in nicotine-induced labeling of ANX7, respectively (*bars 6* and *7*).

We further examined whether phosphorylation of ANX7 *in vivo* could be correlated with catecholamine secretion under the above conditions. Unlabeled cells were pre-incubated for 1 hr in the presence or absence of 100 nM staurosporine, 50 nM calphostine C or 0.7 μM chelerythrine. The cells were then stimulated with or without 100 nM PMA, 100 μM or 10 μM nicotine for 30 min. After incubation, the medium from each well was collected and assayed for secreted catecholamines. As shown in Fig. 1*AII* and 1*BII*, incubation with PMA, carbachol or nicotine results in a 3.5-, 5.3- or 6.0-fold increase in catecholamine secretion, respectively (Fig. 1, *AII* (*bar 2*) and *BII* (*bars 2* and *5*)). By contrast, preincubation with staurosporine, calphostine C or chelerythrine only results in a 1.5-, 1.6- or 1.4-fold increase in PMA-induced secretion, respectively (Fig. 1*AII*, *bars 3-5*). Likewise, calphostine C and chelerythrine also allow 2.7- and 2.9-fold increases in carbachol-induced secretion, respectively (Fig. 1*BII*, *bars 3* and *4*), and 3.1- and 3.2-fold increases in nicotine-induced secretion, respectively (Fig. 1*BII*, *bars 6* and *7*). Moreover, as shown in Fig. 1C, there appears to be a good correlation between the two processes, secretion and ANX7 phosphorylation (R^2 = 0.9622).

In Vitro Phosphorylation of ANX7—To determine whether ANX7 might be a substrate for PKC *in vitro*, we used purified rat brain PKC to phosphorylate purified human recombinant ANX7, and analyzed the products by SDS-PAGE and PhosphorImager. As shown in Fig. 2, PKC indeed phosphorylates ANX7 in a highly efficient manner, and is affected by a variety of extensive variables. ANX7 phosphorylation by PKC is somewhat dependent on pH between pH 6.1 and 7.5 (Fig. 2*A*). After 30 min of incubation at 30^0C, an apparent maximal level of ANX7 phosphorylation by PKC at pH 6.8 is achieved with a stoichiometry of 1.61 $\pm$ 0.13 mole of P_i/ mole of ANX7 (mean $\pm$ S.D., n=5). As compared with the pH 6.8 condition, the stoichiometry of ANX7 phosphorylation at pH 6.1 and 7.5 are 1.35 $\pm$ 0.17 and 1.17 $\pm$ 0.16 mole of P_i/mole of ANX7, respectively. By contrast, the level of autophosphorylation of PKC is relatively unchanged under these pH conditions (*inset* of Fig. 2*A*). Thus, the effect of pH appears to be on the susceptibility of ANX7 to PKC, not on the activity of the PKC, *per se*.

At pH 6.8, phosphorylation of ANX7 is dependent on the presence of PKC, Ca^{+2}, phospholipid, and the PKC activator PMA (Fig. 2*B*). No phosphorylation of ANX7 is detected when PKC is omitted from the reaction mixture (*bar 9*). Similar negative results are found when both Ca^{+2} and phospholipid are omitted from the reaction mixture containing PKC (*bar 1*). Furthermore, the presence of 1 mM Ca^{+2} alone, or phospholipid alone, is unable to support an optimal level of phosphorylation (*bars 2* and *3*). However, when both are present, the level of phosphorylation is greatly enhanced with a stoichiometry of 1.52 $\pm$ 0.02 mole of P_i/mole of ANX7 for 60 min (*bar 4*). Moreover, 100 nM PMA significantly enhances

the level of PKC-catalyzed phosphorylation of ANX7 under the various conditions (compared *bars 5-8* with *bars 1-4*, respectively).

Because we could vary the mole fraction of phosphorylation between 1 and 2, we then examined the kinetics of the process in greater detail (Fig. 2*C*). Under the optimal experimental conditions, phosphorylation of ANX7 is of complete after 60 min with a stoichiometry of 2.01 $\pm$ 0.01 mole of P_i/mole of ANX7 (mean $\pm$ S.D., n=5). The rate of the phosphorylation reaction is also dependent on the ANX7 concentration. As shown in Fig. 2*D*, the efficiency of phosphorylation is decreased as the ANX7 concentration increases. At a higher ANX7 concentration (e.g., 1 μg), however, the optimal level of phosphorylation is achieved only if the incubation time is extended (*inset*).

The fact that Ca^{+2} is absolutely required for ANX7 phosphorylation (see Fig. 2*B*) suggests either that Ca^{+2} is needed to activate only the Ca^{+2}/phospholipid-dependent PKC activity, or that Ca^{+2} binding to ANX7 is a prerequisite for PKC-dependent phosphorylation. To distinguish between these two possibilities, we examined the Ca^{+2} dependence of ANX7 phosphorylation by PKC at both pH 6.1 and 6.8 (Fig. 2*E*). As shown in the *inset* of Fig. 2*E*, the autophosphorylation level of PKC is essentially the same throughout the range of final Ca^{+2} concentrations tested (0.01 to 1.0 mM), at both pH conditions. By contrast, the mole ratio of ANX7 phosphorylation is increased as the Ca^{+2} concentration increases. At pH 6.8, the Ca^{+2}-titration curve for PKC-dependent ANX7 phosphorylation is biphasic. A minimal saturated phosphorylation level with a stoichiometry of 0.5 mole P_i/mole of ANX7 is observed throughout the lower range of Ca^{+2} concentrations (0.01-0.20

mM), and this level is eventually increased as the free Ca^{+2} concentration increases from 0.20 to 1.0 mM. On the other hand, the curve obtained at pH 6.1 is more sigmoidal. Under this pH condition, no phosphorylation of ANX7 is observed at any Ca^{+2} concentration below 0.05 mM, and ANX7 phosphorylation eventually increases as the Ca^{+2} concentration increases beyond 0.05 mM. Thus, the action of Ca^{+2} on ANX7 labeling efficiency appears to be on ANX7 rather than PKC.

Phosphoamino Acid Analysis—To further analyze the PKC reaction both *in vivo* and *in vitro*, we performed phosphoamino acid analysis of ANX7 immunoprecipitated from ^{33}P-labeled chromaffin cells. We also examined ANX7 phosphorylated *in vitro* by purified rat brain PKC. After carbachol or nicotine stimulation, only labeled phosphoserine and phosphothreonine, but not phosphotyrosine, are detected in the immunoprecipitate (Fig. 3*A* and 3*B*). Similarly, acid hydrolysis of ANX7 phosphorylated *in vitro* by PKC only yields labeled phosphoserine and phosphothreonine (Fig. 3*C*). These results thus support the hypothesis that phosphorylation of ANX7 in stimulated chromaffin cells is likely to be mediated by PKC.

Lipid Vesicle Fusion by Phosphorylated ANX7—To study the effect of PKC-dependent phosphorylation on a relevant *in vitro* activity of ANX7, we chose to examine the lipid vesicle fusion reaction mediated by this protein. Two parallel experimental strategies were employed. In one experiment, ANX7 was pre-phosphorylated with PKC in the presence or absence of ATP, followed by extraction

from the reaction mixture, and these phosphorylated and unphosphorylated forms were used to initiate the membrane fusion reaction (Fig. 4*A*). Figure 4*A* shows a time-course of the fusion of lipid vesicles catalyzed by either phosphorylated or unphosphorylated ANX7. When the fusion reaction is initiated with 1 mM Ca^{+2}, the rate and the extent of lipid vesicle fusion induced by phosphorylated ANX7 is markedly enhanced over that of the unphosphorylated ANX7.

In the second experiment, PKC was added simultaneously with ANX7 in the presence or absence of added ATP. As shown in Fig. 4*B*, as the reaction progresses, the relative rate of the fusion reaction containing ATP is greatly enhanced, as compared to that of the control (minus ATP). In parallel control experiments, in which ANX7 has been omitted from the reaction mixture containing either ATP or no ATP, the addition of PKC is unable to induce fusion of lipid vesicles (data not shown). Similarly, the addition of PMA is also unable to alter the fusion reaction induced by ANX7 (data not shown). These two results (Fig. 4*A* and 4*B*) are thus consistent with each other, and suggest that access to phosphorylated ANX7 is a rate-limiting step for efficient activation of membrane fusion.

Ca^{+2} Dependence of Fusion Reactions Induced by Phosphorylated and Unphosphorylated ANX7—Since the fusion of lipid vesicles by ANX7 is dependent on Ca^{+2}, we then examined the effect of phosphorylation by PKC on the Ca^{+2} dependence of this fusion process. In these experiments, phosphorylated and unphosphorylated ANX7 were prepared as described above (see Fig. 4*A*), and their

fusion activities were examined at different final Ca^{+2} concentrations, ranging from 0.01 to 1.0 mM. As shown in Fig. 5*A*, the rate of lipid vesicle fusion induced by phosphorylated ANX7 is markedly increased at lower Ca^{+2} concentrations, as compared with the same process induced by unphosphorylated ANX7 ($p < 0.005$). In addition, not only is the potency of the reaction increased by PKC phosphorylation of ANX7, but the efficacy is also increased. Thus, there is a significant difference between the fusion processes mediated by phosphorylated ANX7 and its unphosphorylated form with the specific consequence of phosphorylation increasing the efficacy and extent of Ca^{+2} activation.

As shown in Fig. 5*B*, the Ca^{+2} concentration required to induce half-maximal fusion activity (50% of F_{max}) is 200 μM for the unphosphorylated protein, which is in accord with previous reports (Caohuy *et al*., 1996; Creutz *et al*., 1978). By contrast, when ANX7 is phosphorylated by PKC, at the same protein concentration, this value is lowered to approximately 50 μM. Thus, Ca^{+2} not only potentiates the susceptibility of ANX7 to labeling by PKC (see Fig. 2*E*), but PKC action also raises the affinity of ANX7 for Ca^{+2}.

It was also possible that the enhancement of fusion by PKC might be due to an increase in lipid binding activity. To test this hypothesis we examined the lipid binding properties of phosphorylated and unphosphorylated ANX7. Following a 20 min fusion reaction, the mixture was centrifuged at 100,000 x g, and the protein bound to lipid vesicles was quantified by SDS-PAGE. The *inset* of Fig. 5*A* shows the recovery of both phosphorylated and unphosphorylated forms of ANX7 from the lipid pellets incubated at different Ca^{+2} concentrations. As shown by the figure,

phosphorylation did not increase the amount of protein recovered with lipid vesicles. Thus, binding of ANX7 to membranes depends exclusively on Ca^{+2}; however, the efficiency by which the Ca^{+2}-induced membrane binding step of ANX7 is converted into membrane fusion depends on PKC.

In Vitro Phosphorylation of ANX7 by Other Kinases and Their Effects on ANX7-driven Lipid Vesicle Fusion—As a control for the specificity of PKC on ANX7 activity, we also examined the phosphorylation of ANX7 by other kinases, including purified PKG, PKA, and $pp60^{c\text{-}src}$, each with an optimal enzymatic activity (Fig. 6*A*). Based on the calculation of ANX7 phosphorylation by these kinases, the amounts of phosphate incorporated into ANX7 were 0.7, 1.0, and 0.9 mole/mole of ANX7 when incubated for 60 min with PKG, PKA, and $pp60^{c\text{-}src}$, respectively (data not shown).

Based on these conditions, we then tested the consequences of phosphorylation by PKA, PKG or $pp60^{c\text{-}src}$ for ANX7 activity on membrane fusion. Using the methods developed to study the PKC effect (see Fig. 4*B*), ANX7 was incubated in the presence of lipid vesicles and PKA, PKG plus cGMP or $pp60^{c\text{-}src}$, with or without added ATP. As shown in Fig. 6*B*, PKA or PKG phosphorylation has no effect on the membrane fusion activity of ANX7. In contrast to the PKC-mediated effect, we observed a modest decrease in the rate of fusion activity in the reaction containing $pp60^{c\text{-}src}$ and ATP.

Discussion

In this study, we present, for the first time, evidence that stimulation of intact bovine chromaffin cells with phorbol ester PMA, carbachol or nicotine markedly increases the phosphorylation of endogenous ANX7 (Fig. 1). Furthermore, using PKC inhibitors with both selective and relatively non-selective properties, we also show that the levels of PKC-dependent labeling of endogenous ANX7 are closely correlated with the levels of catecholamine secretion. These results indicate that ANX7 phosphorylation *in vivo* appears to be mediated by this kinase. Equivalent studies *in vitro* show that ANX7 is a quantitative substrate for PKC (Fig. 2), and that PKC phosphorylation enhances the Ca^{+2}-dependent membrane fusion

reaction driven by ANX7 (Figs. 4 and 5). These findings strongly imply that ANX7 is one of the potential phosphoproteins involved in the exocytotic machinery in chromaffin cells and possibly in other cell types. These conclusions are further supported by our recent report that a nullizygous (-/-) knockout of the anx7 gene in mouse is lethal, and that insulin secretion from islets of Langerhans of the heterozygous knockout anx7 (+/-) mouse is defective (Srivastava *et al*., 1999).

Ca^{+2} and pH Action on ANX7 Control the Efficiency of Phosphorylation by PKC—The limiting factor for the ANX7 phosphorylation event appears to be the structural conformation of the ANX7 protein itself. The efficiency of *in vitro* phosphorylation of ANX7 by PKC is somewhat dependent on pH with an optimal pH of pH 6.8 (Fig. 2*A*). This effect of pH on ANX7 phosphorylation is not attributed to the pH-dependent activity of PKC itself, since the optimal pH of PKC activation is known to be at pH 7.5 (Ferrari *et al*., 1987). Rather, it is likely that ANX7 phosphorylation site(s) become more accessible to PKC at this pH range. Circular dichroism studies of recombinant ANX7 have indicated substantial conformational flexibility over the pH interval of 6.5 to 7.5 (H. B. Pollard, unpublished data). The *in vitro* pH condition (pH 6.8) used to yield an optimal ANX7 phosphorylation appears to be in accord with the cytosolic pH of the chromaffin cell. For instance, several previous studies have shown that the Ca^{+2}-dependent catecholamine secretion is increased at low pH with an optimal pH around pH 6.6 (Knight and Baker, 1985),

and that the cytosolic pH of the chromaffin cell is transiently acidified upon stimulation by acetylcholine or nicotine (Rosario *et al*., 1991).

Our data also support the concept that Ca^{+2} modifies the conformation of ANX7 to permit enhanced labeling by PKC (see Fig. 2*E*). The evidence is that while autophosphorylation of PKC remains unchanged, ANX7 phosphorylation is increased significantly as the free Ca^{+2} concentration elevates from 10 μM to 1 mM. The pH of the medium also dictates the pattern of Ca^{+2}-dependent phosphorylation of ANX7, either having a biphasic dose-response curve (at pH 6.8) or a sigmoidal curve (at pH 6.1). Thus, the elevated Ca^{+2} concentration and the slightly acidic pH, both of which are observed to change coincidentally in the cell during stimulation, appear synergistically to induce the structural conformations of ANX7 that enhance the *in vitro* phosphorylation reaction.

PKC Activates ANX7-driven Membrane Fusion In Vitro—The ANX7-driven membrane fusion reaction is a well-established *in vitro* model for exocytosis (Creutz *et al*., 1982; Hong *et al*., 1982; Nir *et al*., 1987; Pollard *et al*., 1992). The results shown in Figures 4 and 5 suggest that the lipid binding and fusion activities of ANX7 are separable functions, and that only the fusion activity of the protein is regulated by PKC. Phosphorylation of ANX7 by PKC markedly increases the lipid vesicle fusion activity, and significantly lowers the half-maximal Ca^{+2} concentration needed for ANX7-induced lipid vesicle fusion. PKC confers a $K_{1/2, app}$ of 50 μM for phosphorylated ANX7 as opposed to 200 μM for the unphosphorylated form. However, both phosphorylated and unphosphorylated

ANX7 are found to bind to lipid vesicles with equivalent affinities as a function of free Ca^{+2} concentration (*inset* of Fig. 5). Only the phosphorylated protein, however, is able to induce lipid vesicle fusion at lower Ca^{+2} concentrations ($\leq$ 50 μM). At present, the mechanism by which the fusion activity of ANX7 is enhanced by PKC remains to be fully elucidated. Several studies have suggested that annexin self-association, after binding to the membrane, may be required to allow the annexins to aggregate and fuse lipid vesicles (Zaks and Creutz, 1991; Creutz *et al*., 1979). Therefore, it is reasonable to anticipate that phosphorylation of ANX7 by PKC may potentiate the intermolecular interactions occurring between ANX7 molecules, resulting in the enhancement of membrane fusion. ANX7 can therefore be usefully hypothesized as part of the Ca^{+2} control site in the exocytotic machinery. In support of this hypothesis we recall that more than one factor may be involved in mediating the Ca^{+2} signal for exocytosis. For example, while synaptotagmin has been considered as a putative Ca^{+2} receptor for exocytosis (Tahara *et al*., 1998), the knockout mutation of the synaptotagmin gene does not completely abolish Ca^{+2}-evoked secretion (Geppert *et al*., 1994; BeBello *et al*., 1993). By contrast, the anx7 (-/-) knockout is lethal, while the anx7 (+/-) heterozygote expresses only low amounts of ANX7 protein and defectively secretes insulin (Srivastava *et al*., 1999). The lethality of the anx7 (-/-) nullizygous knockout thus serves to emphasize how critical ANX7 is to survival. The secretory defect of the heterozygote anx7 (+/-) animal serves to emphasize that the anx7 gene is critical for the secretory process in some tissues.

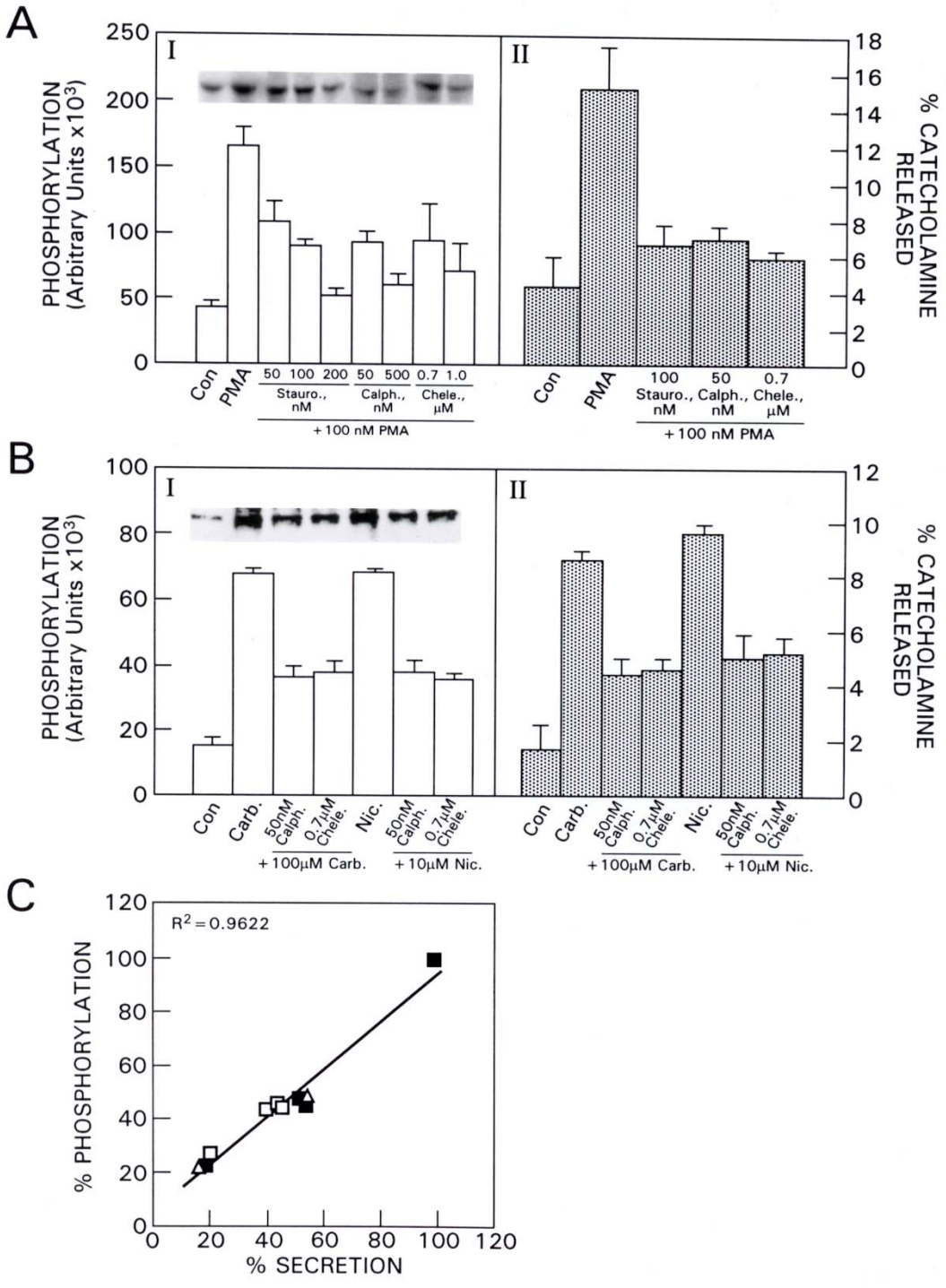
Other Kinases Phosphorylate ANX7, But Do Not Activate Membrane Fusion—Despite ANX7 phosphorylation occurring during a secretion-related phosphorylation event, we have not concluded that PKC is solely responsible for ANX7 phosphorylation in stimulated chromaffin cells. In this report, we show that ANX7 also serves as a good substrate for PKG, PKA, and $pp60^{c\text{-}src}$ *in vitro* (Fig. 6*A*). However, phosphorylation by these kinases either have no significant impact on ANX7-induced lipid vesicle fusion, as seen in the cases of PKA and PKG, or even decrease the rate of fusion of lipid vesicles by ANX7, as seen in the case of $pp60^{c\text{-}src}$. These results thus suggest that, in the presence of a secretion-related phosphorylation event, the fusion activity of ANX7 may be not activated by any of these kinases. This suggestion is supported by previously published reports. For example, in permeabilized chromaffin cells, cAMP (Bittner *et al*., 1986; Knight and Baker, 1982) and cGMP (Knight and Baker, 1982; O'Sullivan and Burgoyne, 1990) have little or no effect on Ca^{+2}-dependent catecholamine secretion. Thus, the direct involvement of PKA or PKG in exocytosis would appear to be ruled out. Similarly, $pp60^{c\text{-}src}$ appears not to be directly involved in exocytosis, because its activity is found to decrease following stimulation of the intact cell (Ely *et al*., 1990). Thus, while ANX7 is an efficient substrate for these kinases, we can only conclude that these kinases may be involved in regulating other as yet unidentified activities of ANX7. Recently, ANX7 has been shown to be phosphorylated by Src kinase, *in vitro* (Furge *et al*., 1999) and to be a tumor suppressor gene for prostate cancer (Srivastava *et al*., 2001).

In conclusion, we have demonstrated that ANX7 serves as the substrate for PKC and certain other kinases. Only PKC-dependent phosphorylation has a positive effect on the *in vitro* membrane fusion model of exocytosis. The specific action involves lowering the $k_{1/2, app}$ for Ca^{+2} from 200 μM to 50 μM. Consistently, stimulation of chromaffin cells with PKC activators indeed results in phosphorylation of endogenous ANX7 concomitantly with the release of catecholamines. These results thus support the hypothesis that ANX7 is a site of action for PKC activation during exocytosis.

Figure 1. **Effects of secretagogues, PKC activator and inhibitors on ANX7 phosphorylation and secretion of catecholamines from chromaffin cells.** ***AI*** and ***BI***, ^{33}P-labeled cells were stimulated for 30 min at 37^0C with 100 nM PMA, 100 μM carbachol, 10 μM nicotine or buffer B (control). In experiments using PKC inhibitors, labeled cells were preincubated for 60 min with or without staurosporine, calphostine C or chelerythrine at indicated concentrations, followed by stimulation with PMA, carbachol or nicotine. The cells were lysed in lysis buffer. The lysates, with equal amounts of total protein, were then incubated with anti-ANX7 antibody for 6 hr at 4^0C. By Western blot analysis, equal amounts of the protein were found to

be present on the blots under the various experimental conditions. Immunoprecipitation of ANX7 was analyzed by SDS-PAGE and PhosphorImager. The level of ^{33}P incorporation into ANX7 is reported as the arbitrary unit (mean ± S.D., n=3-4; *open bars*). The *inset* shows a representative phosphorImager data. ***AII*** and ***BII***, to determine catecholamine secretion under the above conditions, parallel experiments were carried out using unlabeled chromaffin cells, and the media were collected for measuring catecholamine concentrations. The release is expressed as % of the total catecholamine content (mean ± S.D., n=4; *speckled bars*). ***C***, correlation between ANX7 phosphorylation and catecholamine secretion from chromaffin cells in response to various PKC inhibitors and PMA (*empty squares*), carbachol (*filled squares*) or nicotine (*empty triangles*) is shown. Correlation coefficient (R^2) and computer fitted line for all data points are obtained from the results described in Fig. 1*A* and 1*B*.

Figure 1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II
Con
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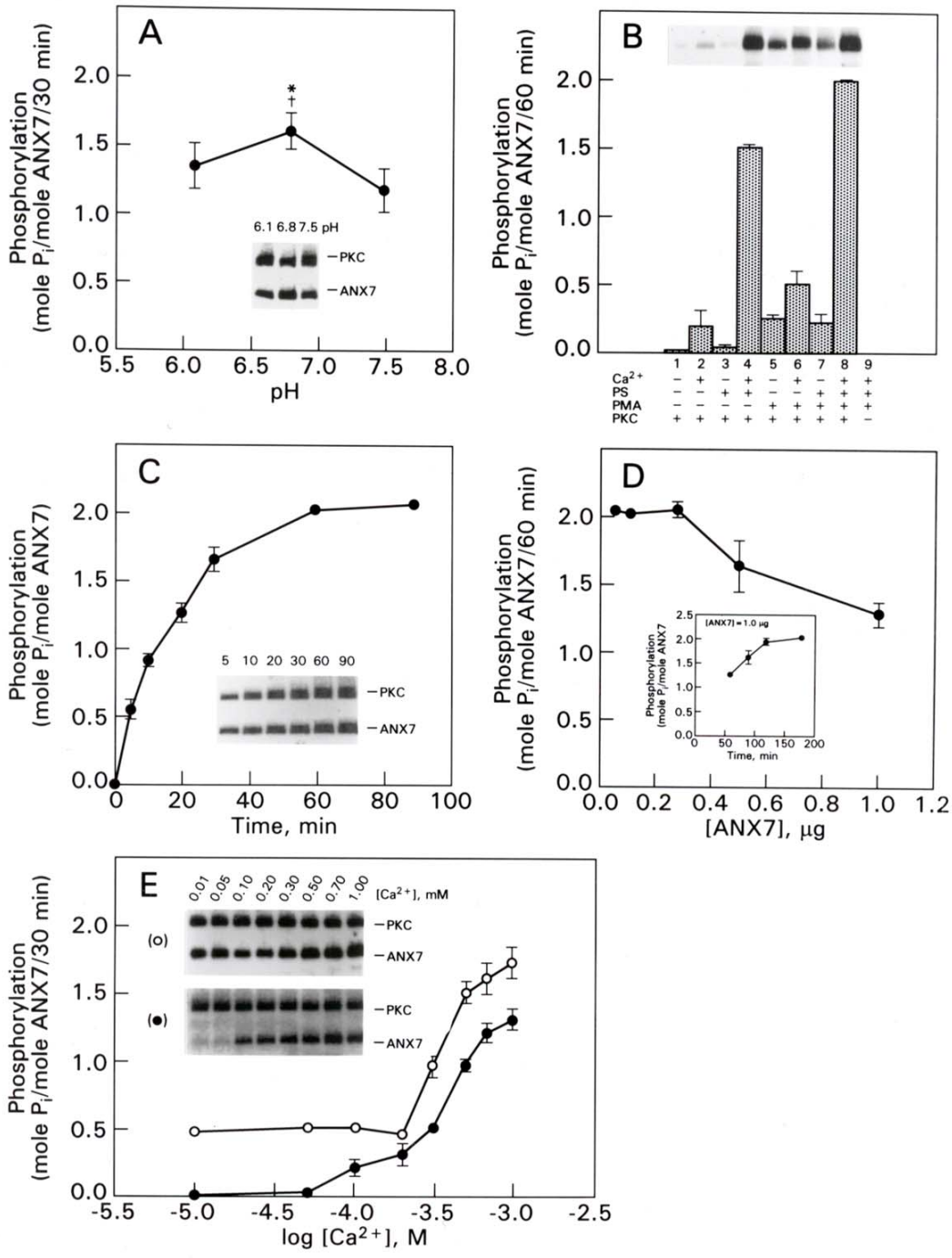
Stauro., nM
Calph., nM
Chele., μM
+100 nM PMA
100 50 0.7
Stauro., Calph., Chele.,
nM nM μM
B
Con
Carb.
50nM Calph.
0.7μM Chele.
Nic.
+100μM Carb.
+10μM Nic.
C
R² = 0.9622
% PHOSPHORYLATION
% SECRETION

Figure 2. ***In vitro*** **phosphorylation of ANX7 by protein kinase C.** ***A***, pH dependence. ANX7 (0.25 μg) was incubated at 30^0C with 0.05 unit of PKC in 25 mM Tris-HCl (pH 7.5), 25 mM PIPES (pH 6.8) or 25 mM MES (pH 6.1), 10 mM $MgCl_2$, 1 mM $CaCl_2$, 100 nM PMA, 400 μg/ml PS liposomes, and 100 μM [γ-^{33}P]ATP. ∗, $p < 0.05$, compared with pH 6.1; †, $p < 0.005$, compared with pH 7.5. ***B***, ANX7 (0.25 μg) was incubated with 0.05 unit of PKC in 25 mM PIPES (pH 6.8), 10 mM $MgCl_2$, 100 μM [γ-^{33}P]ATP, and various conditions as described in the text. ***C***, ANX7 (0.25 μg) was incubated with 0.05 unit of PKC in 25 mM PIPES (pH 6.8), 10 mM $MgCl_2$, 1 mM $CaCl_2$, 100 nM PMA, and 400 μg/ml PS liposomes. The reactions were stopped at the indicated time intervals after the addition of 100 μM [γ-^{33}P]ATP. ***D***, various indicated ANX7 concentrations were incubated for 60 min with 0.05 unit of PKC under the optimal phosphorylation condition (pH 6.8) in a final volume of 30 μl. The *inset* shows the extended time course of phosphorylation of ANX7 at 1.0 μg. ***E***, ANX7 (0.25 μg) and PKC (0.05 unit) were incubated for 30 min in the pH 6.1 (*filled circles*) or pH 6.8 (*empty circles*) phosphorylation buffer containing the indicated free Ca^{+2} concentrations. In *panels A*, *B*, *C*, and *E*, the *inset* shows a representative phosphorImager data of 3-5 different experiments. All data are the mean ± S.D. (n=3-5) and are expressed in mole ratio.

Figure 2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pH
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PKC
ANX7
B
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Ca2+
PS
PMA
PKC
C
Phosphorylation (mole Pi/mole ANX7)
Time, min
5 10 20 30 60 90
D
Phosphorylation (mole Pi/mole ANX7/60 min)
[ANX7], µg
[ANX7] = 1.0 µg
Time, min
E
Phosphorylation (mole Pi/mole ANX7/30 min)
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(o)
(●)

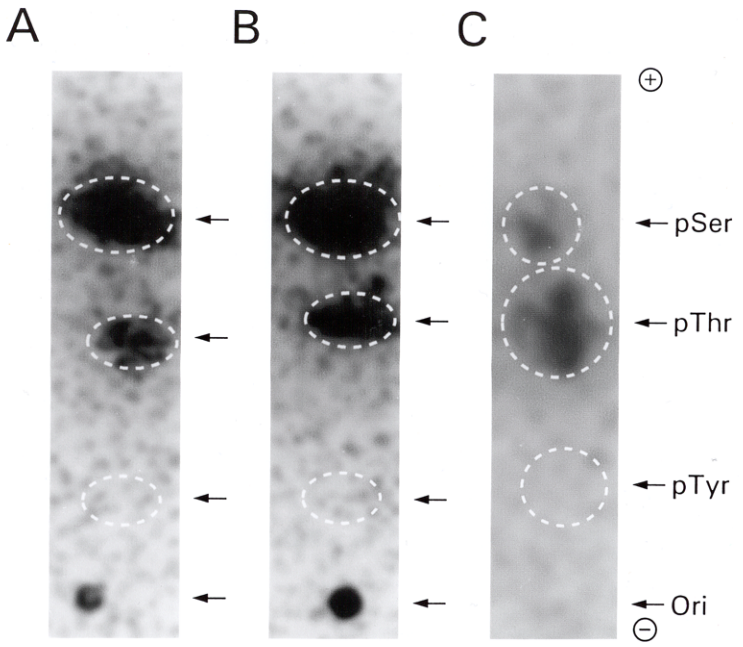


Figure 3. **One-dimensional phosphoamino acid analysis of ANX7.** After autoradiography, the bands of ANX7 immunoprecipitated from stimulated chromaffin cells (*panels A* and *B*) or phosphorylated *in vitro* by PKC (*panel C*) were excised from the gel, and the phosphoamino acid analysis was carried out as described under "Experimental Procedures". *Panel A*, stimulation with 100 μM carbachol. *Panel B*, stimulation with 10 μM nicotine. *Panel C*, *in vitro* phosphorylation by PKC. The positions of the standard phosphoamino acids are outlined by the broken ovals: phosphoserine (P-Ser), phosphothreonine (P-Thr), and phosphotyrosine (P-Tyr). O marks the position of the sample origin.

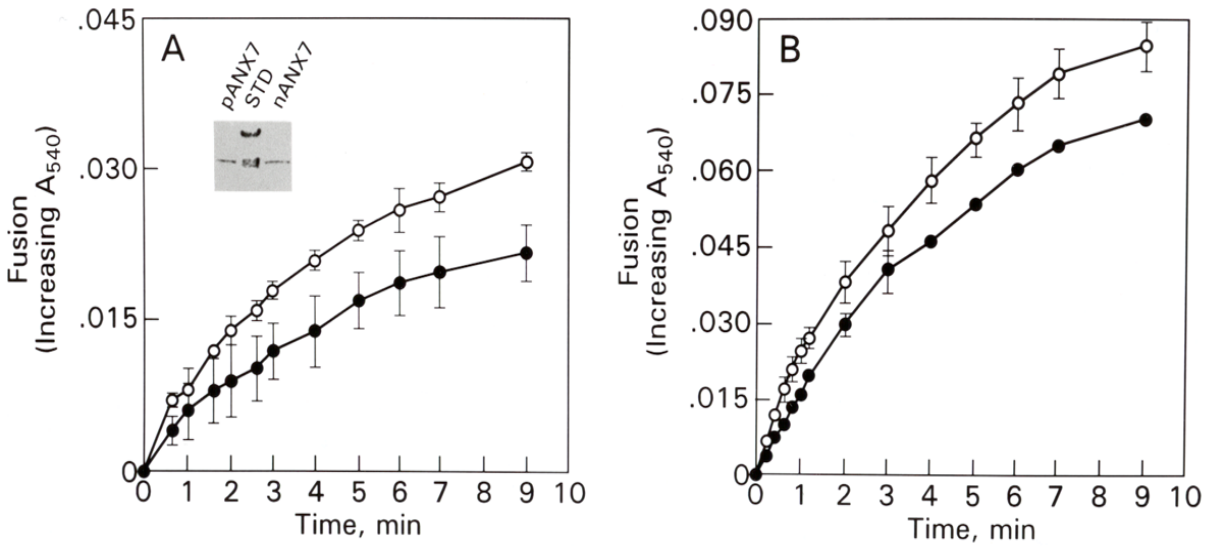


Figure 4. **Effect of PKC-dependent phosphorylation on ANX7-induced lipid vesicle fusion.** ***A***, phosphorylated (pANX7; *empty circles*) or unphosphorylated (nANX7; *filled circles*) ANX7 (0.5 μg), prepared as described under "Experimental Procedures", was added to a 1-ml reaction mixture containing 0.3 M sucrose, 40 mM histidine (pH 6.1), 0.5 mM $MgCl_2$, 0.1 mM EGTA, and 0.5 ml of lipid vesicle suspension. Fusion was initiated by the addition of 1 mM Ca^{+2} and measured by the change in absorbance at 540 nm after 20 min. The *inset* shows the equivalent amounts of the protein present in both reactions. ***B***, ANX7 (1 μg) and PKC (0.5 unit) were added to a 1-ml reaction mixture containing 0.3 M sucrose, 40 mM histidine (pH 6.1), 2 mM $MgCl_2$, 100 nM PMA, 100 μM ATP, and 0.5 ml of lipid vesicle suspension. The control was carried out in the absence of added ATP. Phosphorylation and fusion reactions were simultaneously initiated by the addition of 1 mM Ca^{+2} at room temperature. Fusion was measured by the change in absorbance at 540 nm after 30 min in the presence (*empty circles*) or absence (*filled circles*) of ATP. All data are the mean ± S.D. (n=3).

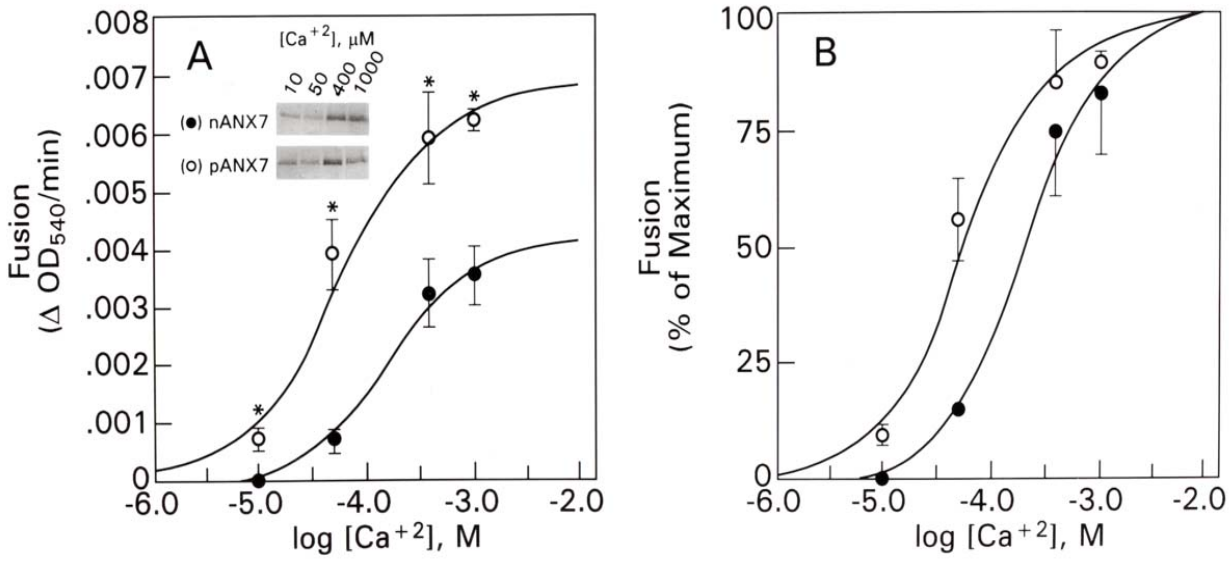
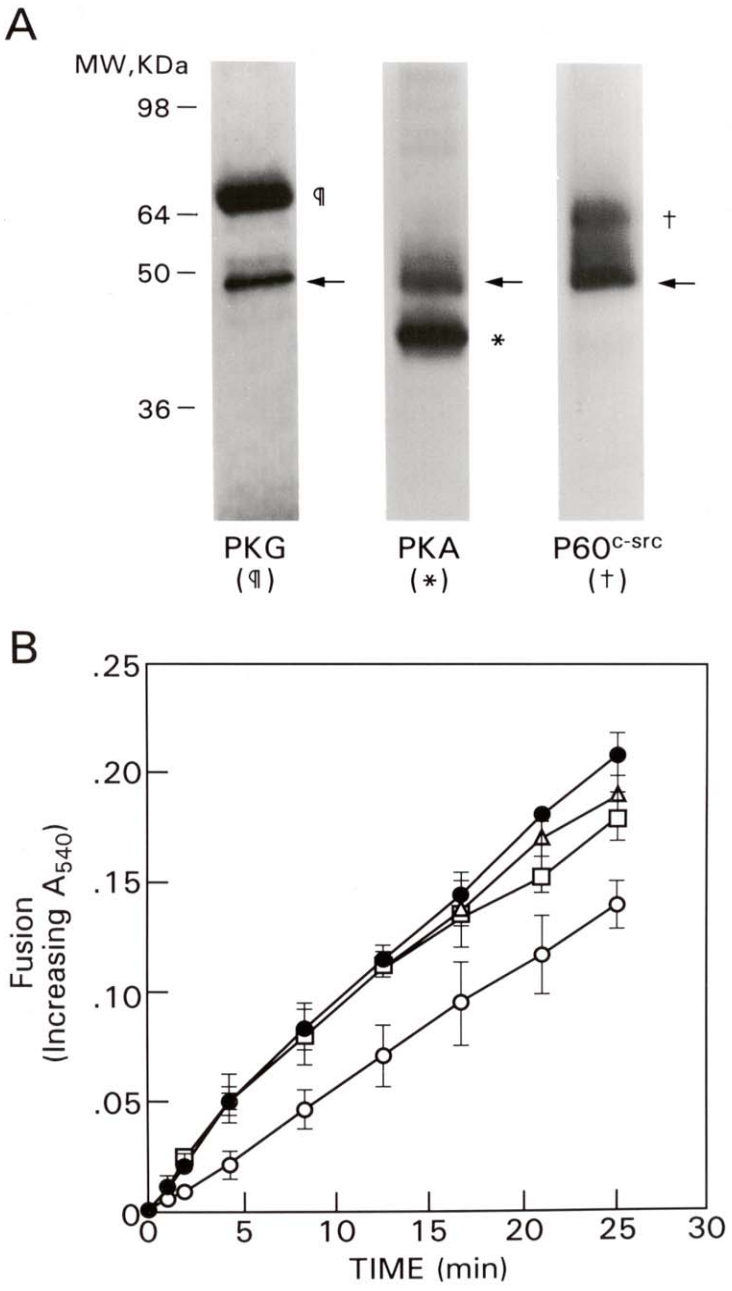


Figure 5. **Ca^{+2} dependence of fusion of lipid vesicles by phosphorylated and unphosphorylated ANX7.** ***A***, phosphorylated (*empty circles*) or unphosphorylated (*filled circles*) ANX7 (0.5 μg), prepared as described in Fig. 4A, was added to a 1-ml reaction mixture containing 0.3 M sucrose, 40 mM histidine (pH 6.1), 0.5 mM $MgCl_2$, 0.1 mM EGTA, and 0.5 ml of lipid vesicle suspension. Fusion was initiated by the addition of the indicated final Ca^{+2} concentrations and measured by the change in absorbance at 540 nm after 20 min. Data are the mean ± S.D. (n=3). ∗, $p < 0.005$, compared with the control (nANX7). To determine lipid binding by ANX7, the lipid vesicles were centrifuged after the fusion reaction was complete, and the lipid-associated protein was analyzed by SDS-PAGE. The *inset* shows phosphorylated ANX7 (pANX7; o) and its unphosphorylated form (nANX7; •) that co-sedimented with the lipid vesicles at indicated free Ca^{+2} concentrations. ***B***, data from *panel A* were replotted to highlight the increasing affinity of ANX7 for Ca^{+2} by PKC phosphorylation. The V_{max} of each curve was determined by a Lineweaver-Burke plot and then used as 100% maximal fusion activity. Based on these V_{max} values, the original data were transformed, expressed as % of maximum of fusion activity and replotted as shown.

Figure 6. ***In vitro*** **phosphorylation of ANX7 by various kinases and their effects on the fusion activity of ANX7.** ***A***, ANX7 (0.25 μg) was incubated for 30

min at 30^0C with 200 units of PKG plus 10 μM cGMP, 50 units of catalytic subunit of PKA or 10 units of $pp60^{c\text{-}src}$ in 25 mM MES (pH 6.1), 10 mM $MgCl_2$, 1 mM $CaCl_2$, and 100 μM [γ-^{33}P]ATP. The result shown is a representative phosphorImager data of 4 different experiments. *Arrows* indicate the position of ANX7. Other labeled bands shown are PKG (¶), PKA_{cat} (*), and $pp60^{c\text{-}src}$ (†). ***B***, ANX7 (1 μg) and 2000 units PKG plus 10 μM cGMP (*empty triangles*), 500 units PKA_{cat} (*empty squares*) or 100 units $pp60^{c\text{-}src}$ (*empty circles*) were added to a 1-ml reaction mixture containing 0.3 M sucrose, 40 mM histidine (pH 6.1), 2 mM $MgCl_2$, 100 μM ATP, and 0.5 ml of lipid vesicle suspension. Controls were carried out in the absence of added ATP (*filled circles*). Phosphorylation and fusion reactions were initiated simultaneously by the addition of 1 mM Ca^{+2} at room temperature. Fusion was measured by the change in absorbance at 540 nm after 30 min. Data are the mean ± S.D. (n=3).

Figure 6



CHAPTER 3

Protein Kinase C and Guanosine Triphosphate Combine to Potentiate Calcium-dependent Membrane Fusion Driven by Annexin 7

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Abstract

Exocytotic secretion is promoted by the concerted action of calcium, guanine nucleotide and protein kinase C. We now show that the calcium-

dependent membrane fusion activity of annexin 7 *in vitro* is further potentiated by the combined addition of guanine nucleotide and protein kinase C. The observed increment involves the simultaneous activation of annexin 7 by these two effectors. Guanosine triphosphate (GTP) and its non-hydrolyzable analogues optimally enhance the phosphorylation of annexin 7 by protein kinase C *in vitro*. Reciprocally, phosphorylation by protein kinase C significantly potentiates the binding and hydrolysis of GTP by annexin 7. Only protein kinase C-dependent phosphorylation has a significant positive effect on annexin 7 GTPase, although other protein kinases, including cAMP-dependent protein kinase, cGMP-dependent protein kinase, and $pp60^{c\text{-}src}$, have been shown to label the protein with high efficiency. *In vivo*, the ratio of bound GDP/GTP and phosphorylation of annexin 7 change in direct proportion to the extent of catecholamine release from chromaffin cells in response to stimulation by carbachol, or to inhibition by various protein kinase C inhibitors. These results thus lead us to hypothesize that annexin 7 may serve as a common site of action for calcium, guanine nucleotide and protein kinase C in the exocytotic membrane fusion process in chromaffin cells.

Introduction

Guanosine triphosphate (GTP) and its non-hydrolyzable analogues (i.e. GTPγS and GMPPNP) are known to promote Ca^{2+}-dependent exocytotic secretion from chromaffin cells and many other cell types (Barrowman *et al*.,

1986; Howell *et al*., 1987; Haslam and Davidson, 1984; Luini and DeMatteis, 1990; Vallar *et al*., 1987; Oetting *et al*., 1986; Knight and Baker, 1985; Bittner *et al*., 1986; Bader *et al*., 1989; Ahner-Hilger *et al*., 1992). Likewise, activation of protein kinase C (PKC) has been shown to trigger Ca^{2+}-dependent secretion in these secreting cell types as well (Pocotte *et al*., 1985; Brocklehurst *et al*., 1985; TerBush and Holz, 1986; TerBush *et al*., 1988; Coorsen *et al*., 1990; Smolen *et al*., 1989; Smolen and Sandborg, 1990; Stojikovic *et al*., 1991; Persaud *et al*., 1989; Wollheim and Regazzi, 1990; Howell *et al*., 1990; Churcher and Gomperts, 1990; Sloan and Haslam, 1987; Walker and Watson, 1993). Furthermore, many permeabilized cell studies have also supported a role for PKC in further enhancing the stimulatory action of Ca^{2+} and GTP in the exocytotic process (Bader *et al*., 1989; Smolen and Sandborg, 1990; Sloan and Haslam, 1997; Cockcroft *et al*., 1987; Ahnert-Hilger *et al*., 1987; Lillie and Gomperts, 1991; Koopman and Jackson, 1990; Carroll *et al*., 1990). These observations thus have lead to the proposal of a hypothetical exocytotic model by Gomperts and his colleagues in which Ca^{2+}, GTP and PKC act in concert in a regulatory sequence leading to exocytosis (Lillie and Gomperts, 1993). In this model, two GTP-binding proteins are involved in controlling the stimulus-secretion process. The first GTP-binding protein is the putative receptor-linked G-protein (G_P) that controls the activity of phospholipase C, thereby generating inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). Further downstream from the signal transduction level, a second GTP-binding protein (G_E, E for exocytosis), a putative GTPase so far undefined as a molecular entity, acts in parallel, or might

be closely associated with a Ca^{2+}-binding protein at the docking/fusion site of the exocytotic machinery. Subsequent phosphorylation by DAG-activated PKC triggers these proteins into mediating the exocytotic membrane fusion process. Although phenomenologically well known, the specific sites of action of Ca^{2+}, GTP and PKC in the stimulus-secretion cascade remain unknown.

Annexin 7 (ANX7; synexin) is a Ca^{2+}-dependent membrane fusion protein (Creutz *et al*., 1982; Hong *et al*., 1982; Nir *et al*., 1987; Pollard *et al*., 1992), for which recent evidence has strongly suggested the possibility of its involvement in exocytosis. For example, we have reported that ANX7 is a Ca^{2+}-activated GTPase, both *in vitro* and in secreting chromaffin cells, and that *in vitro* membrane fusion activity of ANX7 is further enhanced upon binding to GTP (Caohuy *et al*., 1996). More recently, we have reported that the heterozygous knockout Anx7 (+/-) mouse suffers from an insulin secretion deficit from islets of Langerhans, as well as defective Ca^{2+} signaling processes in β-cells (Srivastava *et al*., 1999). Furthermore, we have reported that ANX7 is phosphorylated by PKC, both *in vitro* and in secreting chromaffin cells (Caohuy and Pollard, 2001). Phosphorylation by PKC significantly potentiates the ability of ANX7 to fuse phospholipid vesicles, and the apparent $K_{1/2}$ of Ca^{2+} is lowered from 200 μM to 50 μM (Caohuy and Pollard, 2001). Sequence and site- directed mutagenesis studies of ANX7 have shown that putative binding sites for GTP are located in proximity to consensus phosphorylation sites for PKC. These data have thus led us to hypothesize that these two processes may modulate each other's action in activating ANX7-driven membrane fusion. To test this hypothesis, we have

investigated the interconnections between PKC and GTP action on the Ca^{2+} dependence of ANX7-driven membrane fusion both *in vitro* and *in vivo*.

In this study, we report that GTPγS and PKC both mutually enhance the binding of each other to ANX7, and also potentiate Ca^{2+}-dependent membrane fusion driven by ANX7. *In vitro*, phosphorylation of ANX7 by PKC is optimally enhanced by GTP and its non-hydrolyzable analogues. Reciprocally, the binding and hydrolysis of GTP by ANX7 are markedly potentiated by PKC-catalyzed phosphorylation. While certain other kinases label ANX7 efficiently, they do not substitute for PKC in potentiating GTP binding or membrane fusion. *In vivo*, we find that for ANX7, both the ratio of bound GDP/GTP as well as phosphorylation by PKC change in proportion to the extent of catecholamine release from stimulated chromaffin cells. Thus, GTP and PKC combine specifically to transform ANX7 into a highly efficient Ca^{2+}-dependent membrane fusogen. These *in vitro* data are complemented by *in vivo* data from secreting chromaffin cells. We therefore conclude that the membrane fusion machinery might include ANX7 as a common site of action for Ca^{2+}, GTP and PKC in the exocytotic membrane fusion process.

Experimental Procedures

Preparation of Phosphatidylserine Lipid Vesicles—PS lipid vesicles were prepared fresh daily by the swelling method (Reeves and Dowben, 1968). Highly purified (> 99%) brain phosphatidylserine (Avanti Polar Lipids) in a 1:4 chloroform-methanol solution was dried slowly under nitrogen and then allowed to swell in 0.3 M sucrose at room temperature. The suspension was then

sonicated and centrifuged at 12,000 x g. The PS lipid vesicle pellet was resuspended in sucrose solution.

Isolation and Purification of Human Recombinant ANX7—Human recombinant ANX7 was isolated and purified as described (Burns *et al*., 1990). Briefly, *Escherichia coli* bacteria containing the ANX7-expressing vector (pTrc-FLS) were grown in 1 liter of Luria Broth at 37^0C. After incubation overnight with 1 mM IPTG (ICN), the bacteria were harvested by centrifugation. Expressed recombinant ANX7 was then extracted from the *E. coli* paste, concentrated by precipitation with 0-20% (w/v) $(NH_4)_2SO_4$ and purified by gel filtration using Ultragel AcA54 (Biosphere). This partially purified ANX7 preparation was further purified by binding to PS lipid vesicles in the presence of Ca^{+2} and extracting with EGTA. This purification step was repeated six times to finally yield a highly purified ANX7 preparation ($\geq$ 98%) determined by SDS-PAGE and silver staining.

Lipid Vesicle Fusion Mediated by ANX7—Simultaneous phosphorylation and phospholipid vesicle fusion reactions were carried out as described (Caohuy and Pollard, 2001). The reactions in a final volume of 1 ml contained 1 μg ANX7, 0.5 unit PKC, 0.3 M sucrose, 40 mM histidine (pH 6.1), 2 mM $MgCl_2$, 100 nM PMA, 100 μM ATP, 0.5 ml of lipid vesicle suspension, and with or without 100 μM GTPγS. The controls were carried out in the absence of ATP and/or GTPγS, or in the presence of 500 μM GDPβS. Fusion and phosphorylation were simultaneously initiated by the addition of 1 mM $[Ca^{+2}]_{final}$ at room temperature.

Fusion was measured by the change in the turbidity in absorbance at 540 nm (A_{540}) over a 30-min period using a recording Hewlett-Packard spectrophotometer. The final Ca^{+2} concentration was verified using a Ca^{+2}-selective electrode.

Fusion and phosphorylation reactions in the presence of other protein kinases were carried out as described for the above PKC experiments, except that no PMA was added. PKC was replaced by 2000 units PKG (plus 10 μM cGMP), 500 units PKA_{cat}, or 100 units $pp60^{c\text{-}src}$. These conditions resulted in mole ratios of P_i to protein of 1.0 (Caohuy and Pollard, 2001).

In Vitro Phosphorylation of ANX7—Phosphorylation assays using purified rat brain PKC were performed as described (Caohuy and Pollard, 2001). Rat brain PKC, with a purity of "≥ 95%" and containing isoforms α, β, and γ, was purchased from Calbiochem. To determine the effects of guanine nucleotides on ANX7 phosphorylation, 1 μg of ANX7 was incubated at 30^0C for 1 hr with 0.05 unit (0.035 μg) PKC in a final volume of 30 μl reaction buffer. This buffer consisted of 25 mM PIPES (pH 6.8), 10 mM $MgCl_2$, 1 mM $CaCl_2$, 100 nM PMA, 400 μg/ml PS liposomes, and contained one of the following nucleotides, each with a concentration of 100 μM: GTP, GTPγS, GMP-PNP, or GDPβS. For the time course, 1 μg of ANX7 and 0.05 unit PKC were incubated at 30^0C for indicated time periods in the presence or absence of 100 μM GTPγS. To determine ANX7 phosphorylation as a function of GTPγS concentration, 1 μg of ANX7 was incubated at 30^0C for 1 hr with 0.05 unit PKC in the presence of

various concentrations of GTPγS, as indicated in the figure legend. The controls were carried out in the absence of guanine nucleotides. All reactions were initiated by the addition of 100 μM [γ-^{33}P]ATP (3000-4000 cpm/pmol; Amersham) and terminated by the addition of the SDS-PAGE sample buffer. The reaction products were analyzed by SDS-PAGE and phosphorimaging (PhosphorImager, Molecular Dynamics).

Extraction of Phosphorylated and Unphosphorylated ANX7— Phosphorylation and extraction of the PKC phosphorylated and unphosphorylated protein from lipid vesicles was performed exactly as previously described (Caohuy and Pollard, 2001). Phosphorylated ANX7 has a molar ratio of P_i to protein of 2.0.

GTP Binding—GTP binding of ANX7 was determined with the photoaffinity labeling assay using 8N_3GTP[γ-^{32}P] as described (Caohuy *et al*., 1996), with minor modifications. ANX7 (1 μg) and PKC (0.05 unit) were simultaneously incubated at 30^{0}C in 25 mM PIPES (pH 6.8), 10 mM $MgCl_2$, 1 mM $CaCl_2$, 100 nM PMA, 400 μg/ml PS liposomes, 8 μM 8N_3GTP[γ-^{32}P] (ICN; 10 μCi/mmol), and with or without 100 μM ATP in a final volume of 30 μl. At the indicated times, 4 mM glutathione was added to each sample, and the samples were irradiated for 30 sec at room temperature, followed by SDS-PAGE and phosphorimaging analysis.

GTPase Activity—Assay of ANX7 GTPase was carried out simultaneously with the phosphorylation reaction, and the hydrolytic products were assayed as described (Caohuy *et al*., 1996). To determine the effect of phosphorylation by PKC on ANX7 GTPase activity, 1 μg of ANX7 was incubated at 30^0C for indicated times with or without 0.05 unit PKC in 25 mM PIPES (pH 6.8), 10 mM $MgCl_2$, 1 mM $CaCl_2$, 100 nM PMA, and 400 μg/ml PS liposomes, in a final volume of 30 μl. To determine the GTPase activity as a function of ANX7 concentration, the indicated concentrations of ANX7 were incubated at 30^0C for 1 hr with or without 0.05 unit PKC in the same reaction condition as above. The controls were carried out in the absence of PKC and ANX7, or in the presence of PKC alone. All reactions were initiated by the addition of 100 μM ATP and 50 μM [α-^{33}P]GTP (2000-3000 cpm/pmol; Amersham), and terminated by the addition of 10 μl of 0.5 M EDTA. The reactions (1-μl aliquot) were resolved by thin layer chromatography on polyethyleneimine-cellulose plates (Merck) in 1 M LiCl/1 M formic acid. GTP hydrolysis was assessed by quantitating the formation of [α-^{33}P]GDP with a phosphorimager. The results, after subtracting the background, are calculated as total GDP formed. Background is obtained from the reactions containing PKC alone and without ANX7 and PKC.

In other GTPase assays, phosphorylated ANX7 and its unphosphorylated forms, at protein ratios of 3:0, 2:1, 1.5:1.5, 1:2, or 0:3, respectively, were incubated at 30^0C for 1 hr in the phosphorylation buffer (without ATP, PMA, and PS liposomes) containing 50 μM [α-^{33}P]GTP (2000-3000 cpm/pmol). Each reaction contained the same amount of total ANX7 protein (0.75 μg/ 30 μl

reaction). GTP hydrolysis was assessed by chromatography on PEI plates as described above.

To determine ANX7 GTPase in the presence of other kinases, ANX7 (1 μg) was incubated at 30^0C for 1 hr with or without 200 units PKG (plus 10 μM cGMP), 50 units PKA_{cat}, or 10 units $pp60^{c\text{-}src}$ in 25 mM MES (pH 6.1), 10 mM $MgCl_2$, 1 mM $CaCl_2$, 100 μM ATP, and 50 μM [α-^{33}P]GTP. The controls were carried out in the absence of the respective kinases and ANX7, or in the presence of the kinase alone. GTP hydrolysis was analyzed as described above.

Isolation and Culture of Chromaffin Cells—Chromaffin cells were isolated from bovine adrenal glands by collagenase digestion and purified on Percoll gradient, as described (Caohuy and Pollard, 2001). Isolated cells were further purified by a selective plating method (Yanagihara *et al*., 1979) and maintained in a CO_2 incubator under 5% CO_2/95% air.

[^{33}P] Orthophosphoric Acid Labeling and Treatment of Chromaffin Cells with Carbachol and PKC Inhibitors—Cultured chromaffin cells ($5x10^6$/dish, Falcon, 35 mm) were labeled with [^{33}P] P_i (0.2 mCi/ml; Amersham) in phosphate-free Eagle's MEM medium containing 10% dialyzed fetal calf serum for 10 hr at 37^0C (Caohuy and Pollard, 2001). The cells were washed once with Ca^{+2}-free extracellular buffer A [118 mM NaCl, 4.2 mM KCl, 10 mM $NaHCO_3$, 10 mM glucose, 25 mM Hepes (pH 7.2), 0.1% bovine serum albumin, and 1.2 mM $MgCl_2$]. The cells were pretreated for 1 hr at 37^0C with or without 50 nM

calphostine C (Calbiochem) or 0.7 μM chelerythrine chloride (Calbiochem) in buffer A and then stimulated to secrete by incubation for 30 min at 37^0C in the presence or absence of 100 μM carbachol (Sigma) in extracellular buffer B (buffer A with 2.2 mM $CaCl_2$ added). The control experiments were performed using cells incubated with buffer B or with buffer B containing Me_2SO_4. The latter solvent was a necessary control for drugs such as calphostine C and chelerythrine chloride, which are soluble in Me_2SO_4. After incubation, the media were collected for measuring catecholamine secretion. The cells were rapidly washed twice and then lysed in lysis buffer for immunoprecipitation.

Immunoprecipitation of [^{33}P]-Labeled ANX7—The cells were lysed in 1 ml of ice-cold lysis buffer as described (Caohuy and Pollard, 2001). After clarification by centrifugation, the resulting lysates were precleared by incubation for 30 min with 50 μl of a 10% (v/v) suspension of protein G-Sepharose (Zymed), followed by centrifugation. The final lysates, with equal protein amounts determined by the BCA method (Pierce), were incubated with 10 μg of anti-ANX7 monoclonal antibody 10E7 for 6 hr at 4^0C. The immunoprecipitates were divided into two equal halves, one for determining ANX7 phosphorylation levels and one for assaying GTP/GDP bound to ANX7. All immunoprecipitates were collected on protein G-Sepharose and washed four times by pelleting in ice-cold lysis buffer.

Determination of ANX7 Phosphorylation and Bound GDP/GTP to ANX7— In the assay to determine the extent of ANX7 phosphorylation, the imunoprecipitates were subjected to SDS-PAGE and then electrophoretically transferred to PVDF membranes. Radioactively labeled ANX7 was analyzed by phosphorimaging. To determine the amounts of ANX7 present in the immunoprecipitates, the same membranes were blotted with a polyclonal antibody against ANX7. The bound primary antibody was detected using a peroxidase-conjugated secondary antibody and visualized chromographically using 4-chloro-1-naphthol. In the assay to determine the bound GDP/GTP to ANX7 (Caohuy *et al*., 1996; Downward *et al*., 1990), the immunoprecipitates were incubated for 20 min at 68^0C in elution buffer [25 mM Tris-HCl (pH 7.5), 2 mM EGTA, 2 mM DTT, 0.2% SDS, 0.5 mM GTP, 0.5 mM GDP], followed by centrifugation at 12,000 x g for 15 min at 4^0C. The supernatants were collected, concentrated by lyophilization, and then resuspended in distilled water of a final volume of 5 μl. The entire samples were spotted on a thin layer cellulose plate (Merck), followed by chromatography in 1M LiCl:1M formic acid buffer. Radioactive labeled nucleotides were analyzed by phosphorimaging.

Measurement of Catecholamine Release—The assay for catecholamine release from chromaffin cells was performed exactly as described previously (Caohuy and Pollard, 2001). The release of catecholamines was expressed as a total amount released into the medium.

Statistical Analysis—Data are presented as means ± standard deviation (S.D.) of the mean. A relationship between catecholamine secretion and ANX7 phosphorylation and its guanine nucleotide binding profile was assessed by a linear regression analysis (left and right *y* axes, mean values of the ratio of ANX7-bound GDP/GTP and ANX7 phosphorylation, respectively, induced by carbachol and inhibited by PKC inhibitors; *x* axis, mean value of catecholamine secretion under similar conditions as above). The statistical significant values (*p*) were determined by Student's t test, and a *p* value less than 0.05 was considered significant.

Results

Effects of GTPγS and PKC-Dependent Phosphorylation on ANX7-driven Membrane Fusion Activity—Several studies have shown that Ca^{+2}-dependent secretion is further enhanced by the combined action of guanine nucleotides and PKC *in vivo* (Bader *et al*., 1989; Smolen and Sandborg, 1990; Sloan and Haslam, 1997; Cockcroft *et al*., 1987; Ahnert-Hilger *et al*., 1987; Lillie and Gomperts, 1991; Koopman and Jackson, 1990; Carroll *et al*., 1990). In addition, we have previously reported that GTP (Caohuy *et al*., 1996) and PKC (Caohuy and Pollard, 2001) independently activate the Ca^{+2}-dependent membrane fusion activity of ANX7 *in vitro*. We therefore tested the hypothesis

that the ANX7 membrane fusion activity *in vitro* might be further potentiated by the combined addition of GTP and PKC. To test this possibility, we examined the ANX7-driven lipid vesicle fusion reaction during simultaneous activation of both the phosphorylation of ANX7 by PKC and the presence of GTPγS on ANX7-induced fusion of lipid vesicles. As the fusion reaction progresses in the presence of 1 mM Ca^{+2}, the lipid vesicle fusion activity of ANX7 is indeed activated further by GTPγS-plus-PKC (Fig. 1*A*). The rate and extent of lipid vesicle fusion induced by ANX7 under this condition is significantly increased over that of the control, which contains neither GTPγS nor ATP (Fig. 1*A* & 1*D*, *bars 1 versus 2*; $p < 0.005$). In addition, the increasing ANX7 activity stimulated by the combination of individually optimal concentration of GTPγS and PKC can be distinguished by a simple additive model when comparing activation by either GTPγS or PKC alone (Fig. 1*A* & 1*D*).

Using the above method developed to study the GTPγS-plus-PKC effect, two additional experiments were carried out to test the specificity of this effect on ANX7 membrane fusion activity. In the first set of experiments, an excess molar concentration of the GDP non-hydrolyzable analogue, GDPβS, was added to the phosphorylation-fusion reaction in the presence of both PKC and 100 μM GTPγS. As shown in Fig. 1*B*, the addition of 500 μM GDPβS markedly reduces the lipid vesicle fusion activity of ANX7 stimulated by GTPγS-plus-PKC, and even abolishes the stimulatory effect of PKC on fusion of lipid vesicles driven by ANX7 under this optimal condition. These data are comparable to the finding of inhibition of Ca^{+2}-dependent secretion by GDPβS from various secretory cell

types (Bader *et al*., 1989; Carroll *et al*., 1990; Sontag *et al*., 1992; Raffaniello and Raufmann, 1993; Norman *et al*., 1996; Proks *et al*., 1996; Buccione *et al*., 1994).

In the second set of experiments, we tested the consequences of phosphorylation by PKA, PKG, and $p60^{c\text{-}src}$ for ANX7-driven membrane fusion, in the presence of 100 μM GTPγS. As shown in Fig. 1*C*, the relative rates of lipid vesicle fusion driven ANX7 under these conditions increase moderately, as observed in the case of PKA or PKG, or show no change, as seen in the case of $pp60^{c\text{-}src}$. As compared with the control activity (Fig. 1*D*, *bar 1*), the increasing membrane fusion activities of ANX7 observed here are induced by GTPγS, but not by the phosphorylation catalyzed by these various protein kinases (Fig. 1*D*, *bars 6-8*). The latter conclusion is comparable to our previous reported data demonstrating that GTPγS does enhance ANX7-driven lipid vesicle fusion activity (Caohuy *et al*., 1996), and phosphorylation by either PKA or PKG does not significantly affect this activity, or may even decrease it, as in the case of $pp60^{c\text{-}src}$ (Caohuy and Pollard, 2001). These results thus show that the binding of GTPγS to ANX7, and selective phosphorylation of ANX7 by PKC mutually activate the membrane fusion activity of ANX7 *in vitro*.

Effect of Guanine Nucleotides on Phosphorylation of ANX7 by PKC—To further investigate the mechanism of the additive effect of GTPγS and PKC on ANX7 membrane fusion activity, we examined the effect of GTP and its non-hydrolyzable analogues on the *in vitro* ANX7 phosphorylation reaction. As shown in Fig. 2*A*, the levels of phosphorylation of ANX7 by PKC are optimally

enhanced by GTP and its non-hydrolyzable analogues. At 100 μM, GTPγS significantly increases the level of ANX7 phosphorylation with a stoichiometry of 1.83 ± 0.22 ($p < 0.005$; Fig. 2*A*, *bar 2*) after 1 hr. By contrast, the molar ratio of ANX7 phosphorylation achieved in the absence of GTPγS is 1.27 ± 0.27 (Fig. 2A, *bar 1*), which is in accord with the previously published data (Caohuy and Pollard, 2001). Similarly, GMP-PNP and GTP significantly enhance ANX7 phosphorylation ($p < 0.005$; Fig. 2*A*, *bars 3-4*). However, their effects are less potent than that of GTPγS. At the same concentration as GTPγS, both GMP-PNP and GTP increase the levels of ANX7 phosphorylation with stoichiometries of 1.75 ± 0.2 and 1.64 ± 0.21, respectively, after 1 hr. Strikingly, the order of efficacy, GTPγS > GMP-PNP > GTP, for enhancement of PKC phosphorylation of ANX7 is comparable to that for the stimulation of exocytosis (Howell *et al*., 1987; Haslam and Davidson, 1984; Bittner *et al*., 1986; Bader *et al*., 1989; Raffaniello and Raufmann, 1993). These results thus suggest that, upon binding to GTP and these non-hydrolyzable GTP analogues, ANX7 is configured into a highly susceptible target for phosphorylation by PKC.

As a more definitive test for the positive modulatory role of GTP and its analogues, we determined whether GDPβS could modulate ANX7 phosphorylation by PKC**.** As shown in Fig. 2*A*, GDPβS does not significantly enhance ANX7 phosphorylation by PKC. The molar ratio of ANX7 phosphorylation achieved in the presence of 100 μM GDPβS is 1.32 ± 0.22 (Fig. 2*A*, *bar 5*), which is equivalent to that of the control (Fig. 2*A*, *bar 1*). This finding suggests that although GDPβS binds to ANX7, this type of guanine nucleotide is,

however, incapable of configuring the molecular structure of ANX7. Thus ANX7 is left in the control state.

Furthermore, GTPγS markedly increases the rate and the extent of ANX7 phosphorylation over those of the control (Fig. 2*B*). In the presence of 100 μM GTPγS, phosphorylation of ANX7 catalyzed by PKC is complete after 90 min with a stoichiometry of 1.94 ± 0.10 (n=3). By contrast, in the absence of GTPγS, the optimal level of ANX7 phosphorylation is achieved after 120 min, similar to previously published data (Caohuy and Pollard, 2001). Moreover, the extent of ANX7 phosphorylation modulated by GTPγS is varied depending on the concentrations of this nucleotide. As shown in Fig. 2*C*, the molar ratio of ANX7 phosphorylation is markedly increased in a dose-dependent manner. In the presence of 200 μM GTPγS the level of ANX7 phosphorylation is maximally attained with a stoichiometry of 1.94 ± 0.05 after 1 hr.

In contrast to the increased levels of ANX7 phosphorylation stimulated by GTP and its analogues, the levels of autophosphorylation of PKC are relatively constant under the conditions described above (Figs. 2*A* & 2*C*). Collectively, these results suggest that GTP and its non-hydrolyzable analogues, but not GDPβS, are highly efficient activators of PKC-dependent phosphorylation of ANX7, and that their effects appear to be on the susceptibility of ANX7 to PKC, not on the activity of PKC, *per se*.

Effect of PKC Phosphorylation on Photoaffinity Binding of 8N$_3$GTP[γ-^{32}P] to ANX7 and its GTPase Activity—We next turned our attention to the

question of whether PKC phosphorylation of ANX7 alters guanine nucleotide binding and hydrolyzing activities of ANX7. Previously, we have reported that ANX7 exhibits the ability to bind and hydrolyze intrinsically the bound nucleotides (Caohuy *et al*., 1996). Therefore, we chose to examine both the photoaffinity binding of $8N_3GTP[\gamma\text{-}^{32}P]$ and the intrinsic hydrolysis of $[\alpha\text{-}^{33}P]GTP$ by PKC-phosphorylated ANX7 and its unphosphorylated form. For $8N_3GTP[\gamma\text{-}^{32}P]$ binding assays, ANX7 and PKC were simultaneously incubated at 30^0C in phosphorylation reactions containing $8N_3GTP[\gamma\text{-}^{32}P]$ in the presence or absence of 100 μM ATP, followed by irradiation at the indicated times. Figure 3 shows that incubation of ANX7 with PKC in the presence of ATP significantly stimulates ANX7's ability to bind $8N_3GTP[\gamma\text{-}^{32}P]$ in a time-dependent manner. We calculated from the phosphorimager data that the binding affinity of PKC-phosphorylated ANX7 for $8N_3GTP[\gamma\text{-}^{32}P]$ is increased 3-fold over that for the unphosphorylated form of ANX7. Although this finding clearly shows that ANX7 phosphorylation by PKC significantly enhances the guanine nucleotide binding activity of ANX7, we anticipated that $8N_3GTP[\gamma\text{-}^{32}P]$ could be rapidly hydrolyzed by ANX7 during the reaction. To avoid this hydrolysis problem, we performed a membrane filter binding assay using $GTP\gamma S[S^{35}]$ as a substrate. Consistently, this latter result also shows a substantial increase in the binding of $GTP\gamma S[S^{35}]$ to PKC-phosphorylated ANX7, yielding a binding ratio of 0.081 pmol $GTP\gamma S$/min/ pmol ANX7 (data not shown).

For GTP hydrolysis assays, two parallel experimental strategies were employed. In the first experimental strategy, both ANX7 phosphorylation by PKC

and GTP hydrolysis by ANX7 were initiated simultaneously by the addition of 100 μM ATP and 50 μM [α-^{33}P]GTP. Figure 4 shows the time course of the hydrolysis of [α-^{33}P]GTP by ANX7 in the presence or absence of added PKC. In the presence of added PKC, the intrinsic GTPase activity of ANX7 is significantly increased in a time-dependent manner. Ultimately, the overall rate of GTP hydrolysis catalyzed by PKC-phosphorylated ANX7 is approximately 7-fold faster than that determined for the native form of ANX7. We calculated that the molar turnover number of the steady-state GTPase reaction mediated by phosphorylated ANX7 is 0.086 ± 0.006 pmol GDP/min/pmol ANX7. By contrast, the equivalent value for the native form of ANX7 is 0.012 ± .001 pmol GDP/min/pmol ANX7. Thus, phosphorylation by PKC also modulates ANX7's intrinsic GTPase activity, as it does on ANX7's guanine nucleotide binding activity (Fig. 3).

In the second experimental strategy, ANX7 was incubated at 30^{0}C for 3 hr with PKC in the presence or absence of added ATP, and affinity isolated on PS liposomes. After eluting from the liposomes, the different ANX7 forms were assayed for GTPase activity. Similar to the results obtained from the first experiment (Fig. 4), pre-phosphorylated ANX7 exhibits higher detectable GTPase activity than the unphosphorylated form of ANX7 (Fig. 5; *bars 5 versus 1*). In another experiment, to further examine the relationship between ANX7 phosphorylation by PKC and GTPase activity of ANX7, we mixed pre-phosphorylated ANX7 and its unphosphorylated form at protein ratios of 2:1, 1.5:1.5, or 1:2, respectively. These reactions were then assayed for GTPase

activity. Strikingly, the amounts of GTP hydrolysis are increased linearly with the increasing amounts of pre-phosphorylated ANX7 added (Fig. 5; *bars 3-4*). As shown in the *lower panel* of Fig. 5, immunoblotting analysis using an antibody against ANX7 clearly shows that each GTPase reaction contains approximately the same amount of ANX7 protein. These findings thus are consistent with the results in the first experiment, indicating that phosphorylation by PKC indeed enhances the hydrolysis of GTP by ANX7.

In a parallel control experiment, we tested the possibility that the increasing GTPase activity might be due to a contaminant of the PKC preparation. As shown in the *upper panel* of Fig. 4, addition of PKC alone has no significant effect on GTP hydrolysis over the entire time course. Furthermore, the results shown in Fig. 5 may also eliminate the possibility of a contaminant from the ANX7 sample that might contribute to the increasing GTPase activity of ANX7. Collectively, these findings thus clearly indicate that PKC-catalyzed phosphorylation stimulates both guanine nucleotide binding and hydrolysis activities of ANX7.

Effects of Phosphorylation by Other Protein Kinases on ANX7 GTPase Activity—To determine if the enhancement of the ANX7 GTPase activity was specific for PKC-mediated phosphorylation or was a more general effect of the phosphorylation process, we examined the effects of phosphorylation by other protein kinases on ANX7 GTPase activity. Using the methods developed to study the PKC effect (see Fig. 4), ANX7 was incubated for 1 hr with or without

PKC, PKA, PKG, or pp60$^{c\text{-}src}$ in a phosphorylation reaction containing 50 μM [α-^{33}P]GTP. In contrast to the enhanced effect of PKC phosphorylation on this activity, simultaneous phosphorylation of ANX7 by PKA, PKG, and pp60$^{c\text{-}src}$ do not yield a significant increase above the basal GTPase activity (Fig. 6). Thus, the nature of the phosphorylation process does indeed regulate the specificity of particular kinase's action on ANX7 guanine nucleotide binding/ hydrolysis property.

Catecholamine Secretion, ANX7 Phosphorylation, and ANX7-Bound GDP/GTP in Stimulated Chromaffin Cells—To correlate the *in vitro* data with events in cells, we examined the biochemical profile of endogenous ANX7 in secreting chromaffin cells. In these experiments, intact bovine adrenal chromaffin cells were metabolically labeled with [^{33}P] orthophosphoric acid, and then stimulated with or without 100 μM carbachol. Following cell lysis and immunoprecipitation, both ANX7 phosphorylation and binding of GDP/GTP to ANX7 were analyzed and compared simultaneously with the release of catecholamines into the medium. As shown in Fig. 7*C*, catecholamine secretion in response to carbachol is increased concomitantly with the increasing levels of both the ANX7 phosphorylation and the ratio of ANX7-bound GDP/GTP (Fig. 7*A* & *B*, respectively). By contrast, stimulation of cells with buffer B (control) results in small changes in secretion, ANX7 phosphorylation, and ANX7-bound GDP/GTP.

Furthermore, we examined whether both the *in vivo* phosphorylation of ANX7 and the binding of GDP/GTP to ANX7, along with catecholamine secretion, could be inhibited by the selective PKC inhibitors. For these experiments, labeled chromaffin cells were pretreated with either PKC inhibitor calphostine C (50 nM) or chelerythrine chloride (0.7 μM) prior to incubation with 100 μM carbachol. As shown in Fig. 7*A*-*C*, both PKC inhibitors markedly reduce the levels of catecholamine secretion, ANX7 phosphorylation, and the ratio of ANX7-bound GDP/GTP from cells stimulated with carbachol. These *in vivo* findings thus clearly show a close relationship between catecholamine secretion and ANX7 phosphorylation and its guanine nucleotide binding profile, with correlation coefficient (R^2) of 0.9698 (Fig. 7*D*). These data thus complement the *in vitro* data. Together, the present findings further support the hypothesis that ANX7 functions as a Ca^{+2}/GTP-binding protein/PKC substrate, very close to the exocytotic membrane fusion site in the stimulus-secretion cascade.

Discussion

GTP and PKC, in concert with Ca^{+2}, are known to constitute a highly potent intracellular effector system for exocytosis in a variety of secreting cell types (Barrowman *et al*., 1986; Howell *et al*., 1987; Haslam and Davidson, 1984; Luini and DeMatteis, 1990; Vallar *et al*., 1987; Oetting *et al*., 1986; Knight and Baker, 1985; Bittner *et al*., 1986; Bader *et al*., 1989; Ahner-Hilger *et al*., 1992; Pocotte *et al.*, 1985; Brocklehurst *et al*., 1985; TerBush and Holz, 1986; TerBush *et al*., 1988; Coorsen *et al*., 1990; Smolen *et al*., 1989; Smolen and Sandborg, 1990; Stojikovic *et al*., 1991; Persaud *et al*., 1989; Wollheim and Regazzi, 1990; Howell *et al*., 1990; Churcher and Gomperts, 1990; Sloan and Haslam, 1987; Walker and Watson, 1993; Cockcroft *et al*., 1987; Ahnert-Hilger *et al*., 1987; Lillie and Gomperts, 1991; Koopman and Jackson, 1990; Carroll *et al*., 1990). In addition, Gomperts and his colleagues (Lillie and Gomperts, 1993) have suggested that these effectors may exert their positive actions either directly on a common site, or on putative target proteins that are closely associated with each other in the exocytotic machinery. Based on our previous studies (Caohuy *et al*., 1996; Caohuy and Pollard, 2001), we have proposed that ANX7 might function as a

common site for these effectors in the exocytotic machinery. To further support this hypothesis, we demonstrate here that the Ca^{+2}-dependent lipid vesicle fusion activity of ANX7 *in vitro* is significantly amplified by the combination of GTPγS and PKC (Fig. 1). Furthermore, the *in vitro* data on ANX7 membrane fusion activity appear to be well correlated with what we have observed in the *in vivo* studies. In these *in vivo* studies with ANX7, the ratio of bound GDP/GTP and phosphorylation by PKC change in direct proportion to the extent of catecholamine release from [^{33}P]-labeled chromaffin cells in response to stimulation by carbachol, or to inhibition by various PKC inhibitors. This close correlation between the *in vivo* and the *in vitro* data thus implies that ANX7 functionally behaves like a G_E (G-protein for exocytosis; Lillie and Gomperts, 1993), and transduces the intracellular signals for exocytosis by simultaneously binding GTP and being phosphorylated by PKC in a Ca^{+2}-dependent manner.

Based on these findings, the simplest explanation for the observed additive effects of PKC and GTPγS on ANX7 membrane fusion activity *in vitro* involves the same mechanisms of activation induced by these two agents *in vitro*. Indeed, our further *in vitro* analyses have shown that the combined presence of guanine nucleotides and PKC in the reaction mixture simultaneously increases the sensitivity to each other's action on ANX7.

Guanine nucleotides stimulate ANX7 phosphorylation by PKC—With regard to the PKC action on ANX7, we have found that the efficiency of ANX7 phosphorylation by PKC *in vitro* is further enhanced by GTP and its non-

hydrolyzable analogues, but not by GDPβS (Fig. 2). Significantly, the concentrations of added guanine nucleotides that activate this ANX7 phosphorylation event are relevant to the physiological GTP concentration range (Otero, 1990; Bourne *et al*., 1991). These data clearly imply that the binding of GTP and its non-hydrolyzable analogues to ANX7 can confer conformational flexibility that makes ANX7 phosphorylation sites more accessible to PKC. Since ANX7 is a Ca^{2+}-dependent GTPase (Caohuy *et al*., 1996), this implication appears to be relevant since increased conformational flexibility due to GTP is a common feature of most GTPases. Such flexibility enables GTPase proteins to function as molecular switches in which GTP- and GDP-bound forms have different conformations, and therefore significantly different activities (Melancon, 1993). To further support this concept, we have found that activation of ANX7 phosphorylation by guanine nucleotides is not attributed to changes in PKC activity. The evidence is that the levels of autophosphorylation of PKC, which is proportional to the activity of the kinase (Newton, 1995), remain relatively constant under all experimental conditions tested (Fig. 2*A* & 2*C*). Furthermore, we have found that the rank order of effectiveness for ANX7 phosphorylation by PKC is GTPγS > GMP-PNP > GTP >> GDPβS (Fig. 2*A*). This finding indicates that the binding of GTP rather than its hydrolysis is of critical importance for the phosphorylation process. In addition, the finding indicates that this modification is specifically sensitive to the activated, GTP-bound form of ANX7. Together with the data from our previous published study (Caohuy and Pollard, 2001), the present data strongly suggest that GTP further enhances the synergistic action of

the elevated Ca^{2+} concentration and the slightly acidic pH (i.e. pH 6.8) in transforming ANX7 into a highly susceptible substrate for phosphorylation by PKC. Significantly, this optimal condition for the *in vitro* ANX7 phosphorylation by PKC appears to be physiologically relevant, since all of these factors are observed to be localized endogenously, as in the case of GTP, or to change coincidently, as in the case of Ca^{2+} and pH, in the cell during stimulation.

Phosphorylation by PKC stimulates the ability of ANX7 to bind and hydrolyze GTP—Reciprocally, the ANX7 phosphorylation by PKC substantially stimulates the basal levels of GTP binding and GTP hydrolysis by ANX7 (Figures 3-5). The significance of these results is that upon phosphorylation by PKC, the turnover number for ANX7 is now relatively equivalent to those of some known G-proteins, including EF-G, EF-Tu, tubulin, and the G components of adenylate cyclase and transducin, with turnover numbers of 0.012-0.25 mol/min/mol of protein (Chinali and Parmeggiania, 1980; Fasano *et al*., 1982; Carlier and Pantaloni, 1982; Brandt, 1983; Fung, 1983). Furthermore, the rate of GTP binding for phosphorylated ANX7 is quite similar to that of GTP hydrolysis, indicating that the hydrolytic/exchange reaction is rapid and is limited by GDP dissociation. Such an indication has been supported by the present data (Fig. 1*B*) showing that the addition of excess GDPβS markedly inhibits the additive effect of PKC and GTPγS on ANX7 membrane fusion activity. This result strongly suggests that the exchange reaction of GDPβS for GTPγS is blocked by the excess molar concentration of GDPβS. Thus, it appears from these data that once ANX7-bound GTP is hydrolyzed, the newly formed GDP is released rapidly,

and the empty nucleotide-binding pocket of ANX7 is ready to accommodate a new GTP molecule. ANX7 has a higher affinity for GTP than it has for GDP, thus indicating that the exchange is supported by energetic properties (Caohuy *et al*., 1996).

At present, the mechanisms by which both binding and hydrolysis of GTP by ANX7 are enhanced by PKC phosphorylation remain to be fully elucidated. Nonetheless, it is plausible to speculate that the ANX7 conformational change induced by the PKC phosphorylation event is instrumental for both the stimulated GTPase activity and the rapid GDP/GTP exchange.

No Effects of phosphorylation by other protein kinases on ANX7 GTPase Activity—The *in vitro* studies have shown that, in a simultaneous GTPase-phosphorylation reaction, all PKA-, PKG-, and $pp60^{c\text{-}src}$-catalyzed phosphorylation do not significantly alter the molar turnover number of the GTPase reaction mediated by ANX7 (Fig. 6). These results strongly suggest that, unlike to PKC, these kinases may phosphorylate ANX7 on sites distant to the GTP binding site, and are thus incapable of influencing the binding and hydrolysis of GTP of ANX7. Thus, the lack of stimulation by PKA, PKG, and $pp60^{c\text{-}src}$ on both the guanine nucleotide binding/hydrolysis property and on the membrane fusion activity (Fig. 1*C*) of ANX7 coincides with other observations showing that these kinases are not directly involved in regulated exocytosis (Bittner *et al*., 1986; Cheek and Burgoyne, 1987; Shono *et al*., 1997; Kumai *et al*., 1998; Ely *et al*., 1990; Ohnishi *et al*., 2001). In addition, the lack of effects of

these phosphorylation events, compared to the consequences of PKC, serves as an important control for emphasizing the importance of PKC induced changes in ANX7 function.

Membrane fusion cycle of ANX7—Based on all of the present observations and of those in previous published reports (Caohuy *et al*., 1996; Caohuy and Pollard, 2001), we propose the following ANX7-driven membrane fusion cycle (see Fig. 8). (1) Under a resting, low-Ca^{+2} condition, ANX7 exists in an inactive state [ANX7-Mg^{+2}-GDP], which is formed by a process of constitutive Mg^{+2}-dependent hydrolysis of GTP. (2) Upon elevation of Ca^{+2}, ANX7 binds Ca^{+2}, transforming into a moderate active form [ANX7-Ca^{+2}/Mg^{+2}-GDP], which can drive membrane fusion. (3) GDP bound to this form can be replaced by GTP, and in this [ANX7-Ca^{+2}/Mg^{+2}-GTP] form, membrane fusion activity of ANX7 is further activated. Upon hydrolysis of GTP to GDP, a transient [ANX7-Ca^{+2}/Mg^{+2}-GDP] complex is formed. (4 & 5) Under a suitable phosphorylation condition, either the GDP- or GTP-bound form of ANX7 can be phosphorylated by PKC, and PKC phosphorylation oscillates ANX7 between two phosphorylated states, [**P**-(ANX7-Ca^{+2}/Mg^{+2}-GTP)] and [**P**-(ANX7-Ca^{+2}/Mg^{+2}-GDP)], by stimulating the intrinsic GTPase and GTP/GDP exchange activities of ANX7. As a result, the membrane fusion activity of ANX7, with an order of efficiency [**P**-(ANX7-Ca^{+2}/Mg^{+2}-GTP)] > [**P**-(ANX7-Ca^{+2}/Mg^{+2}-GDP)], is at the optimal level, even operating at lower Ca^{+2} concentrations. (6) The GDP-bound, phosphorylated ANX7 is subsequently dephosphorylated by the action of a serine/threonine protein phosphatase (PPase) which, we have now learned, is

calcineurin (Caohuy and Pollard, unpublished data). Then, with the reduction of free Ca^{+2} concentration, the GDP-bound, unphosphorylated ANX7 releases Ca^{+2} and returns to the inactive form, and the cycle can recur.

In summary, the present observations on ANX7 are remarkably congruent with the original exocytotic model of Gomperts. The ANX7 data are consistent with the concept that the stimulatory actions of Ca^{+2}, GTP and PKC converge on ANX7 to drive membrane fusion activity occurring during exocytosis. To further support such an inference, we have recently found that botulinum neurotoxin type C (BoNT/C), which is a zinc-dependent protease and a specific inhibitor of exocytosis (Dolly *et al*., 1994), efficiently cleaves ANX7 both *in vitro* and in permeabilized chromaffin cells. This proteolytic activity is concurrent with BoNT/C-dependent inhibition of ANX7 membrane fusion activity *in vitro*, and with inhibition of catecholamine secretion *in vivo*, respectively (Caohuy and Pollard, 2002). These recent findings significantly parallel the proteolytic effect of this toxin on syntaxin (Schiavo *et al*., 1995) and SNAP-25 (Blasi *et al*., 1993), which are protein components of the SNARE hypothesis (Rothman and Orci, 1992). Inasmuch as the identification of SNARE proteins as targets for BoNTs has been taken as *prima facie* evidence favoring the SNARE hypothesis for exocytotic membrane fusion, the apparent role of ANX7 in the exocytotic membrane fusion process thus cannot be excluded.

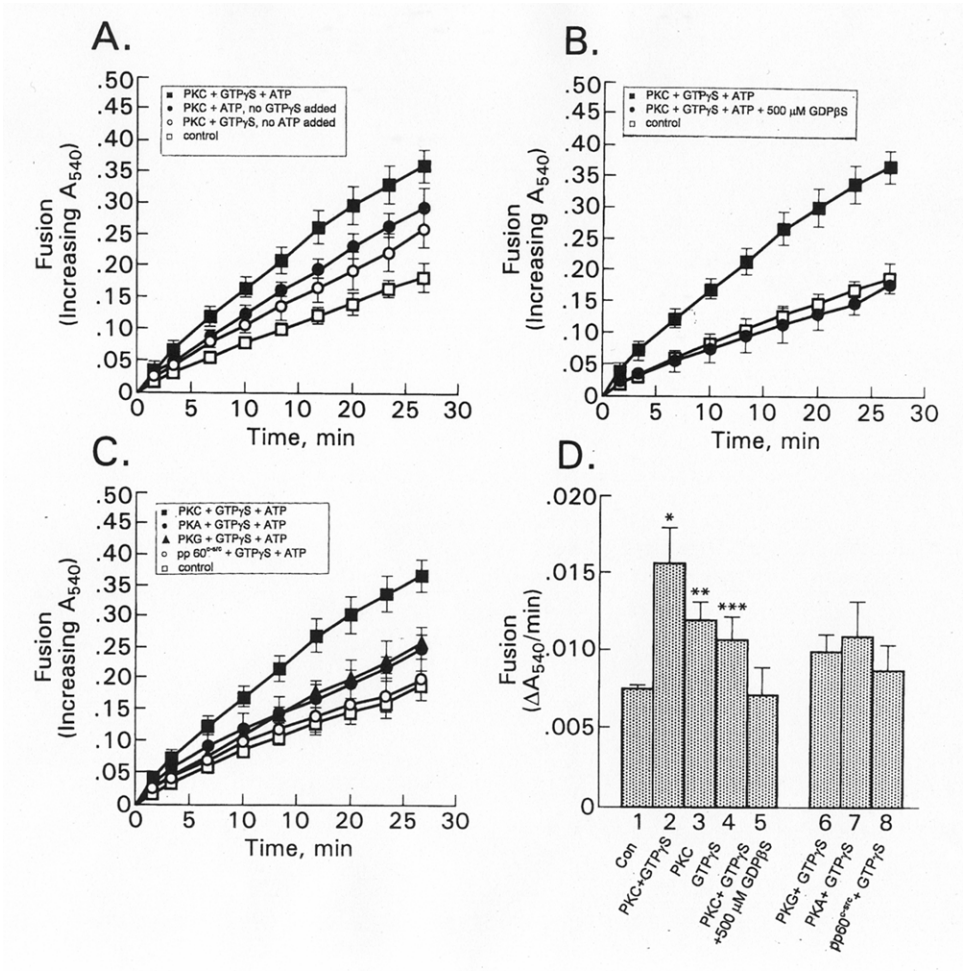


Figure 1. **Effects of guanine nucleotides and phosphorylation by protein kinases on ANX7-driven lipid vesicle fusion.** ***A***, ANX7 (1 μg) and PKC (0.5 unit) were added to a fusion-phosphorylation reaction in the presence (■) or absence (●) of 100 μM GTPγS, or in the presence of GTPγS but no ATP added (○). ***B,*** ANX7 and PKC were added to a reaction mixture containing both 100 μM GTPγS and 500 μM GDPβS (●). ***C,*** ANX7 (1 μg) and PKG (2000 units; ▲), PKA_{cat} (500 units; ●) or $pp60^{c\text{-}src}$ (100 units; ○) were added to a fusion-phosphorylation reaction containing 100 μM GTPγS. ***D*,** the rates of lipid vesicle fusion driven by ANX7 obtained in *A*-*C* are summarized. *Bars 1*, control; *2*, PKC plus GTPγS; *3*, PKC minus GTPγS; *4*, PKC plus GTPγS, no ATP added; *5*, PKC plus GTPγS and GDPβS; *6*, PKG plus GTPγS; *7*, PKA plus GTPγS; and *8*, $pp60^{c\text{-}src}$ plus GTPγS. $*p < 0.005$ and $**p < 0.05$ compared with the control. In *A*-*C*, the reaction containing neither ATP nor GTPγS was used as the control (□). In all panels, the phosphorylation and fusion reactions were simultaneously initiated by the addition of 1 mM Ca^{+2} at room temperature. Fusion was measured by the change in absorbance at 540 nm for 30 min. All data are the means ± S.D. (n=4).

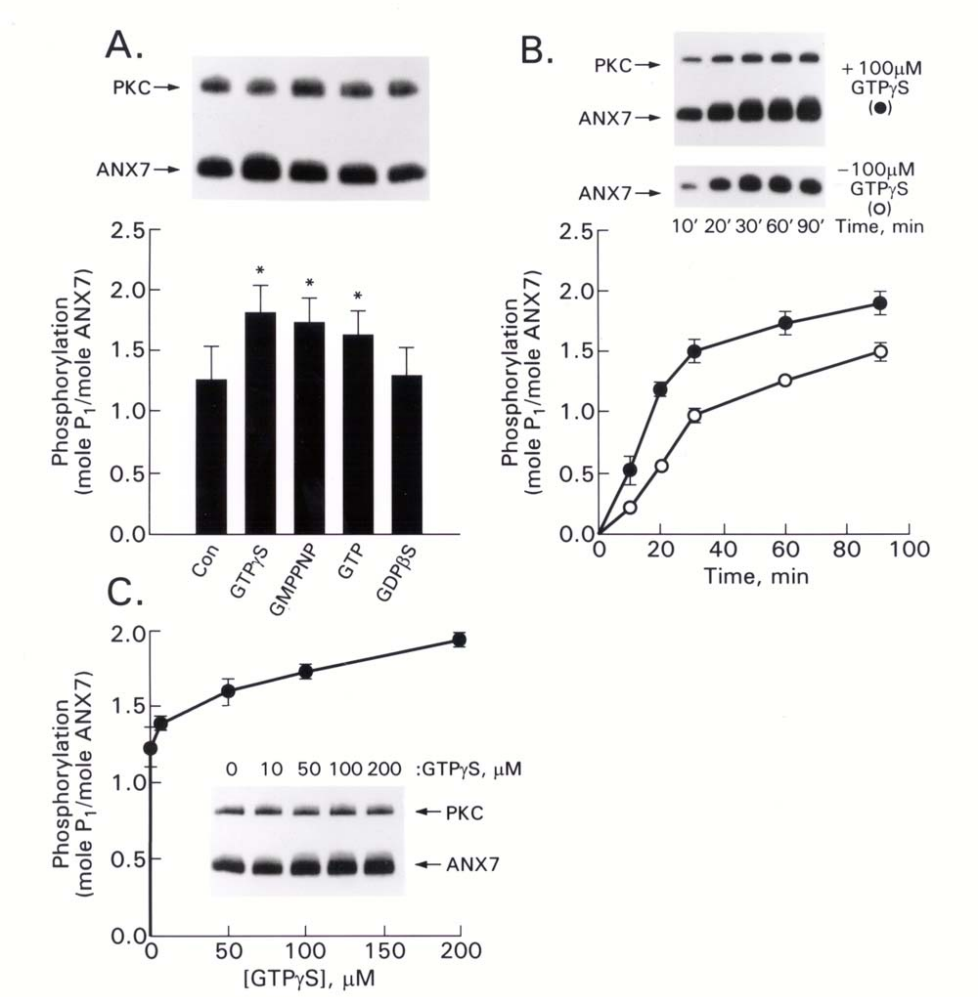


Figure 2. **Effect of guanine nucleotides on phosphorylation of ANX7 by PKC.** ***A****,* the effect of GTPγS, GMP-PNP, GTP, and GDPβS (100 μM each) on ANX7 phosphorylation by PKC. **p* < 0.005. ***B****,* the time course of ANX7 phosphorylation by PKC in the presence (●) or absence (○) of 100 μM GTPγS. ***C****,* PKC-dependent phosphorylation of ANX7 as a function of GTPγS concentration. In all panels, the phosphorylation reaction mixtures, as described under "*Experimental Procedures",* were carried out at 30^0C for 1 hr (*A* & *C*), or various indicated times (*B*), and then analyzed by SDS-PAGE and phosphorimaging. The reaction containing no guanine nucleotide was used as the control. Data are the means ± S.D. (n=4). *Upper panels* (*A* & *B*) and the *insert* (*C*) show the representative phosphorImager data.

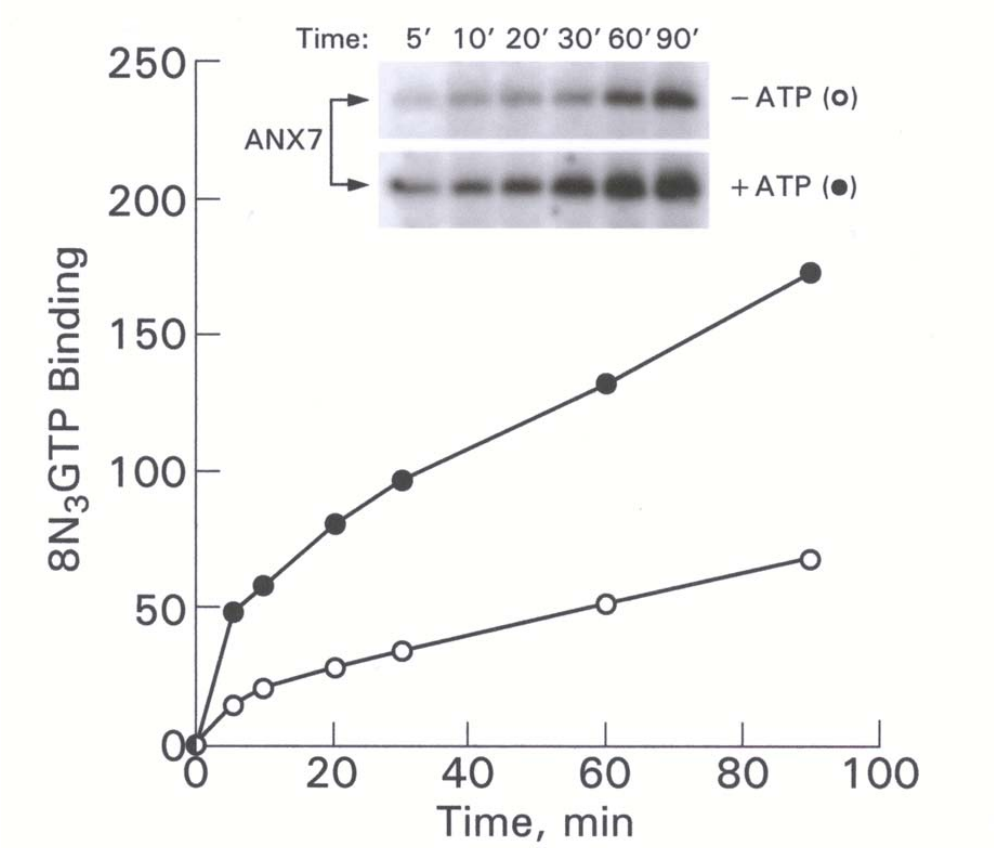


Figure 3. **Effect of PKC phosphorylation on photoaffinity binding of $8N_3GTP[\gamma\text{-}^{32}P]$ to ANX7.** ANX7 (1 μg) and PKC (0.05 unit) were incubated at 30^0C in a phosphorylation reaction containing 8 μM $8N_3GTP[\gamma\text{-}^{32}P]$ in the presence (●) or absence (○) of 100 μM ATP. At the indicated times, the reactions were irradiated for 30 sec at room temperature and subjected to SDS-PAGE analysis. ^{32}P incorporation was analyzed by phosphorimaging. Results are the mean of two independent experiments. The *inset* shows representative phosphorimaging data.

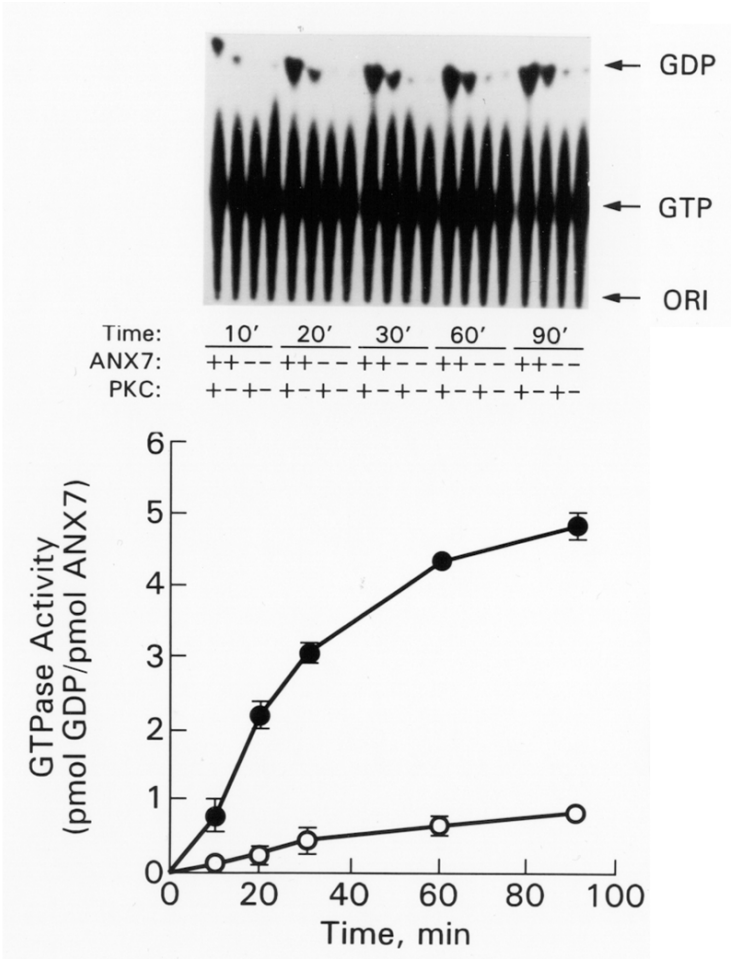


Figure 4. **Effect of PKC phosphorylation on GTPase activity of ANX7.** ANX7 GTPase activity was measured simultaneously with ANX7 phosphorylation by PKC as described under "*Experimental Procedures*". ANX7 (1 μg) was incubated at 30^0C for various indicated times in the presence (●) or absence (○) of 0.05 unit PKC. The reactions were initiated by the addition of ATP and [α-^{33}P]GTP, and the products were analyzed by polyethyleneimine-cellulose thin layer chromatography and phosphorimaging. The positions of GTP, GDP, and origin (ORI) are indicated. Data are the means ± S.D. (n=4). The levels of GTP hydrolysis produced in the reactions containing PKC alone and the buffer alone were subtracted from the values presented. The *Upper panel* shows representative phosphorImager data. *Lane 1*, ANX7 plus PKC; *lane 2*; ANX7 alone; *lane 3*, PKC alone; *lane 4*, buffer.

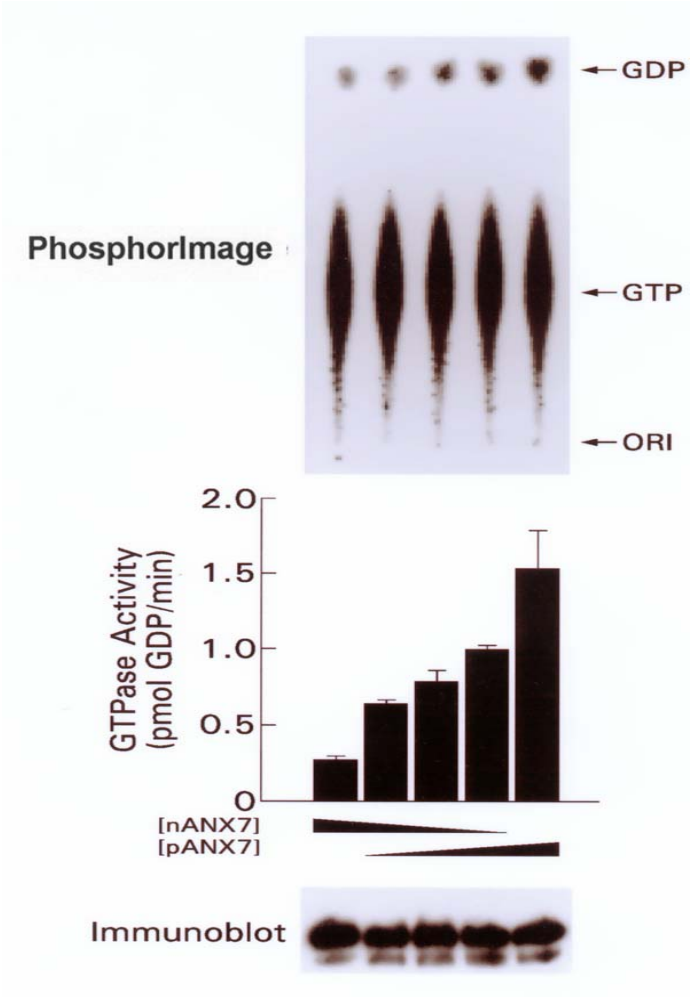


Figure 5. **GTPase activity of PKC-phosphorylated and unphosphorylated forms of ANX7.** PKC-phosphorylated ANX7 and its unphosphorylated form were prepared as described under "*Experimental Procedures*". Mixtures of unphosphorylated ANX7 alone (*lane 1*), phosphorylated ANX7 alone (*lane 5*), or unphosphorylated and phosphorylated forms at protein ratios of 2:1, 1.5:1.5, or 1:2 (*lanes 2-4*, respectively) were incubated at 30^0C for 1 hr. Each mixture contained the same amount of total ANX7 protein (0.75 μg/ 30 μl reaction). The GTPase reaction products were analyzed as described in Fig. 4. The *top panel* shows representative phosphorimaging data. The value of GTP hydrolysis produced in the reaction containing the buffer alone was subtracted from the values presented (mean ± S.D., n=3). The *bottom panel* shows the immunoblot of the above reactions reacted with ANX7 monoclonal antibody.

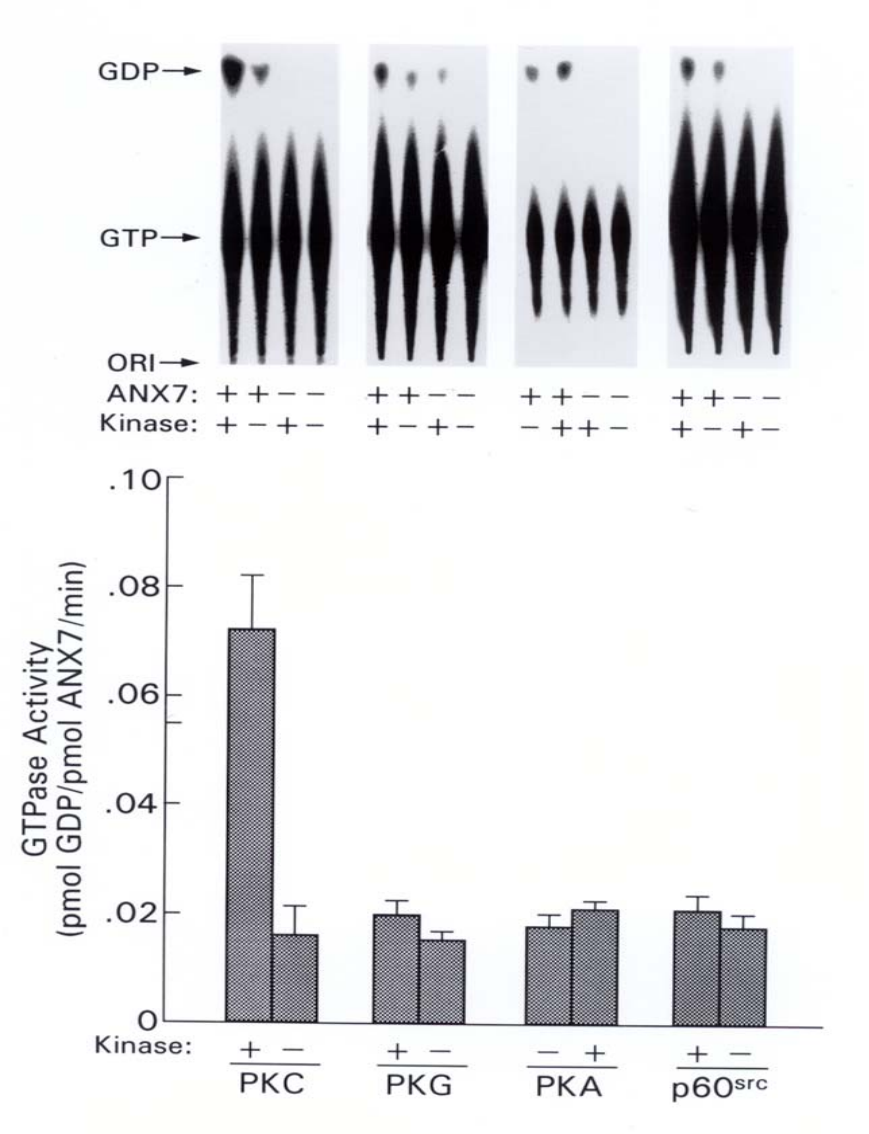
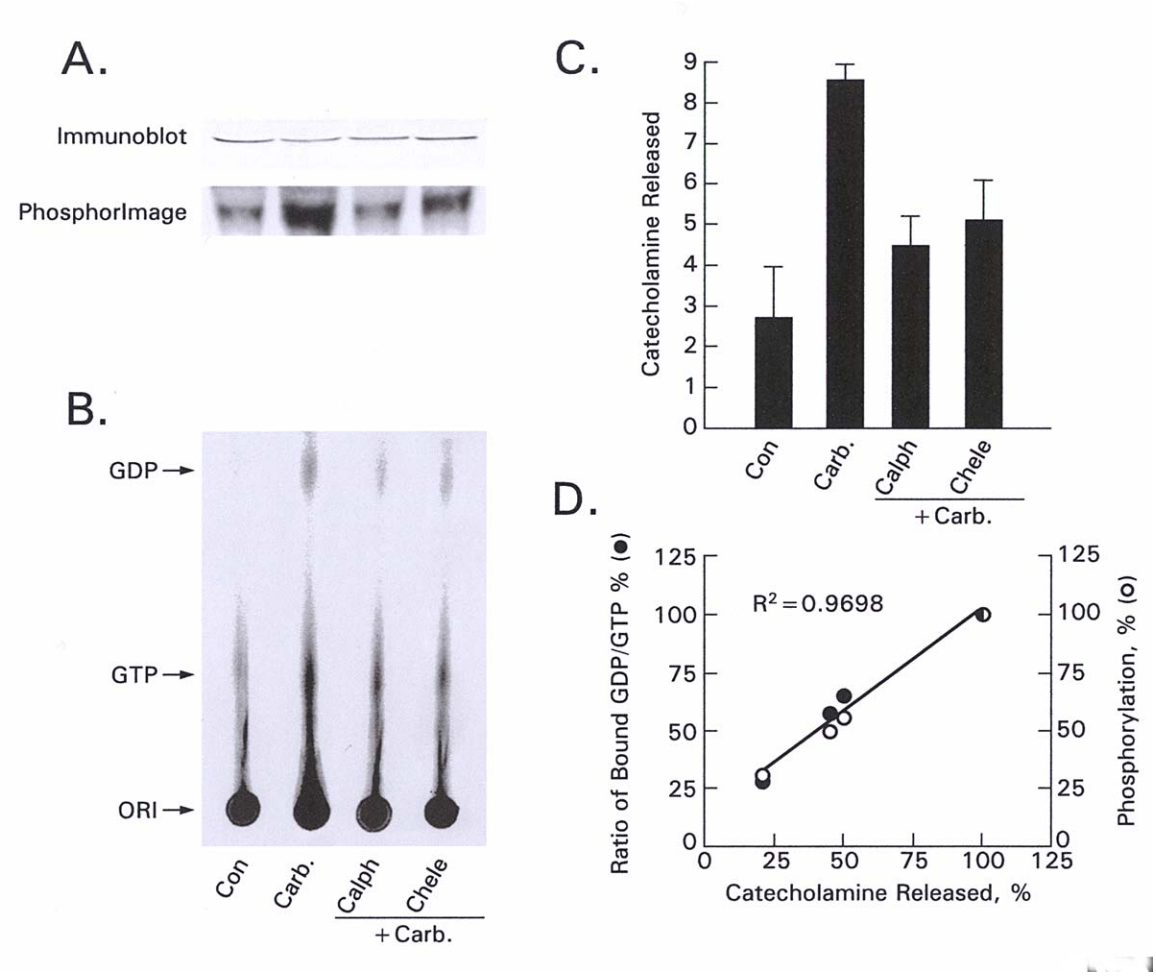


Figure 6. **Effects of phosphorylation by various protein kinases on ANX7 GTPase activity.** The effect of phosphorylation by various protein kinases on ANX7 GTPase activity was determined and analyzed as described in Fig. 4. ANX7 (1 μg) was incubated at 30^0C for 1 hr with or without 200 units PKG, 50 units PKA_{cat}, or 10 units $pp60^{c\text{-}src}$. The values of GTP hydrolysis produced in the reactions containing kinase alone and the buffer alone were subtracted from the values presented. Data are the means ± S.D. (n=4). The *upper panel* shows a representative phosphorImager data. *Lane 1*, ANX7 plus kinase; *lane 2*, ANX7 alone; *lane 3*, kinase alone; *lane 4*, buffer.

Figure 7. **Effects of PKC inhibitors and carbachol on catecholamine secretion, ANX7 phosphorylation, and ANX7-bound nucleotides in cultured chromaffin cells.** The ^{33}P-labeled chromaffin cells were stimulated with or without 100 μM carbachol at 37^{0}C for 30 min, or preincubated with or without indicated PKC inhibitors, followed by stimulation with carbachol. Following cell lysis, the lysates were immunoprecipitated with the anti-ANX7 monoclonal antibody. The immunoprecipitates were divided into two equal halves. The first half of the immunoprecipitate was then analyzed by SDS-PAGE, followed by electrotransfer to a PVDF membrane, for protein phosphorylation, while the second half was analyzed by thin layer chromatography for guanine nucleotide binding. ^{33}P incorporation into ANX7 and labeled GTP/GDP bound to ANX7 were analyzed by phosphorimaging, and the phosphorimaging data represent one of three different experiments. ***A***, the levels of ^{33}P incorporation into immunoprecipitated ANX7. After phosphorimaging, the membrane was immunoblotted with another anti-ANX7 polyclonal antibody, and the immunoreactive ANX7 bands were detected chromogenically (*top panel*). ***B***, the ratio of bound GDP/GTP to immunoprecipitated ANX7. The positions of GDP, GTP, and origin are indicated. ***C***, catecholamines secreted into the medium, from the same cells, were measured and expressed as μg of epinephrine plus norepinephrine (mean ± S.D., n=3). Abbreviations are carb, carbachol; calph, calphostine C; and chele, chelerythrine chloride. ***D***, correlation between catecholamine secretion and ANX7 phosphorylation(○) and ANX7-bound GDP/GTP (●) in response to stimulation by carbachol, or to inhibition by various PKC inhibitors. Correlation coefficient (R^2) and the *computer-fitted line* for all data points were obtained from the results described in *A*-*C*.

Figure 7


A.
Immunoblot
PhosphorImage
B.
GDP
GTP
ORI
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+Carb.
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+Carb.
D.
Ratio of Bound GDP/GTP % (●)
Phosphorylation, % (○)
R² = 0.9698
0
25
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100
125
Catecholamine Released, %

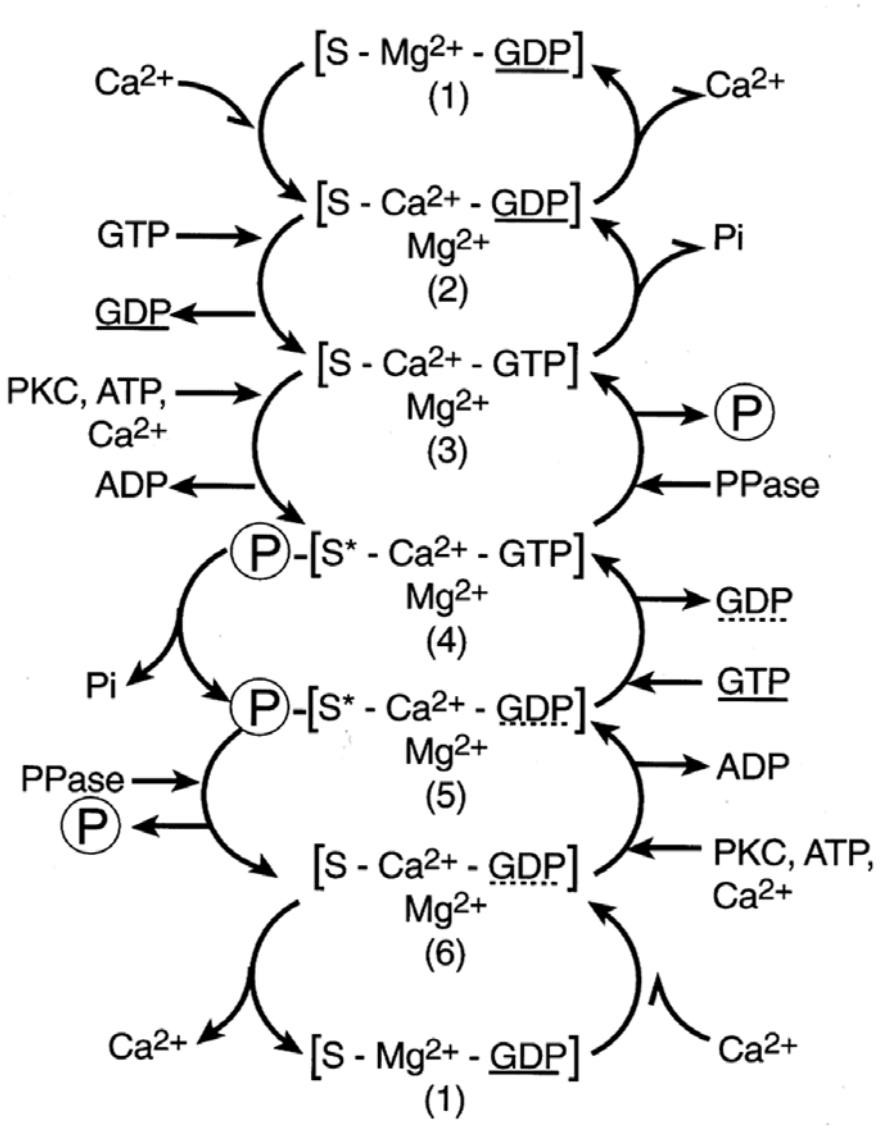

Figure 8. **ANX7 membrane fusion cycle regulated by Ca^{+2}, GTP and PKC.** This model schematically illustrates that ANX7 oscillates between two major transitional states. In the "off" state and low Ca^{+2} concentrations, ANX7 is inactive and exists in the favored GDP-bound form. Upon elevation of Ca^{+2}, GDP bound to ANX7 can be replaced with GTP and this GTP-bound form is optimally phosphorylated by PKC, activating ANX7 (the "on" state), which drives membrane fusion much more efficiently. Then, with the reduction in the free Ca^{+2} concentration, the "off" state complex is reformed, and the cycle can recur. See "*Discussion*" for a detailed explanation.

CHAPTER 4

Annexin 7: A Non-SNARE Proteolytic Substrate for Botulinum Toxin Type C in Secreting Chromaffin Cells

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Abstract

Botulinum toxins (BoNT) are zinc proteases, which specifically cleave proteins involved in regulated exocytosis, and block secretory processes. We show here that treatment of permeabilized chromaffin cells with BoNT/C results in the reduction of annexin 7 immunoreactivity. *In vitro*, BoNT/C cleaves recombinant human annexin 7 with the production of a ≈29-kDa fragment. These findings further support the hypothesis that annexin 7 may be a component of the membrane fusion complex regulating exocytosis.

Introduction

Investigations into the mechanism of the secretory process have been greatly helped by the use of specific inhibitors of exocytosis, particularly botulinum and tetanus toxins (Dolly *et al.*, 1994). Numerous studies have shown that the light chains of these toxins are zinc-dependent proteases, and that the proteolytic activity of these toxins is directed against specific proteins that have been proposed to be essential in exocytosis (Schiavo *et al.*, 1992). Although the identification of VAMP/synaptobrevin, SNAP-25, and syntaxin (Niemann *et al*., 1994) as targets for the BoNTs demonstrates a function in exocytosis, the mechanism underlying the membrane fusion process remains enigmatic.

Annexin 7 (ANX7; Synexin), a Ca^{+2}-dependent membrane fusion protein (Raynal and Pollard, 1994), has properties that have led us to give fuller credence to the possibility of its involvement in exocytosis. We have reported that ANX7 is a Ca^{+2}-activated GTPase (Caohuy *et al*., 1996). Recently, we have reported that ANX7 is phosphorylated by protein kinase C (PKC), both *in vitro* and in activated chromaffin cells, and that only PKC-dependent phosphorylation potentiates the ability of the protein to fuse lipid vesicles and lowers the apparent $K_{1/2}$ of Ca^{+2} (Caohuy and Pollard, 2001). In addition, we noted that ANX7 contains sequences homologous to those hitherto associated with known cleavage sites of BoNTs on SNARE proteins. Therefore, we hypothesize that ANX7 might also be a hitherto unsuspected main target for BoNTs at the exocytotic site in secretory cells.

Experimental Procedures

Bovine chromaffin cells were exposed to digitonin in the presence or absence of DTT-reduced BoNT/C (Calbiochem) (Foran *et al*., 1996), followed by SDS-PAGE and immunoblotting. For *in vitro* proteolytic assays (Schiavo *et al*., 1995), purified recombinant human ANX7 was incubated at 37^0C for 1 hr in the presence or absence of DTT-reduced BoNT/C, followed by SDS-PAGE and immunoblotting. For binding assays, ANX7 or heat-inactivated ANX7 (boiled at 100^0C for 10 min) was incubated at 4^0C for 1 hr in a buffer containing sepharose crosslinked to ANX7, BoNT/C or nothing using the AminoLink kit (Pierce). For fusion assays (Caohuy and Pollard, 2001), toxin-treated or untreated recombinant ANX7 was added to a lipid vesicle fusion reaction. Fusion was measured at A_{540nm} using a recording spectrophotometer.

Results

ANX7 has been shown to serve as a common mediator for the positive effects of Ca^{+2}, GTP and PKC in the lipid vesicle fusion process (Caohuy *et al*., 1996; Caohuy and Pollard, 2001), suggesting that ANX7 may play a role in mediating the membrane fusion process occurring during exocytosis. To further test this hypothesis, we investigated whether ANX7 is the target of BoNTs. In this study, we focused our attention on particularly BoNT/C. As shown in Fig. 1, treatment of permeabilized chromaffin cells with BoNT/C causes a dose-dependent reduction in ANX7 immunoreactivity, but not β-actin immunoreactivity. Consistent with the results reported by other labs, we have found that BoNT/C also causes a reduction in catecholamine secretion (data not shown).

Figure 2 presents the results of an experiment designed to test the effect of BoNT/C on the lipid vesicle fusion activity of ANX7. As shown, fusion activities of toxin-treated ANX7 are significantly reduced as compared to that of untreated ANX7 (Fig. 2*A*), and the activity found in each case is correlated with the amount of intact ANX7 that is bound to lipid vesicles (Fig. 2*B*). We have also found that the immunoreactive levels of intact ANX7 are reduced concomitantly with the appearance of a ≈29-kDa fragment (Fig. 3*A*). Finally, to demonstrate unambiguously that ANX7 and BoNT/C interact, we performed the binding assay. As shown in Fig. 3B, ANX7 indeed binds to sepharose-immobilized BoNT/C. This interaction depends on a native ANX7 conformation, since heat-inactivated ANX7 does not bind to immobilized BoNT/C.

Discussion

This preliminary study is the first demonstration that ANX7 is a substrate for BoNT/C, both *in vitro* and in permeabilized chromaffin cells. The potency and efficacy are similar to those needed to breakdown syntaxin (Foran *et al*., 1996; Schiavo *et al*., 1995) and SNAP-25 (Blasi *et al*., 1993b). Based on the size of the cleavage product, we predict that the cleavage site of BoNT/C on ANX7 is located in the second annexin repeat (Fig. 4). As shown in this figure, this domain contains an amino acid sequence that is closely resembled to that of syntaxin isoforms. These data are further supported by the binding experiment (Fig. 3*B*), which indicates that ANX7 may contain BoNT/C recognition sites. In conclusion, the present findings provide direct evidence supporting the concept that ANX7 may be an important component of the putative vesicle docking and fusion apparatus in regulated exocytosis.

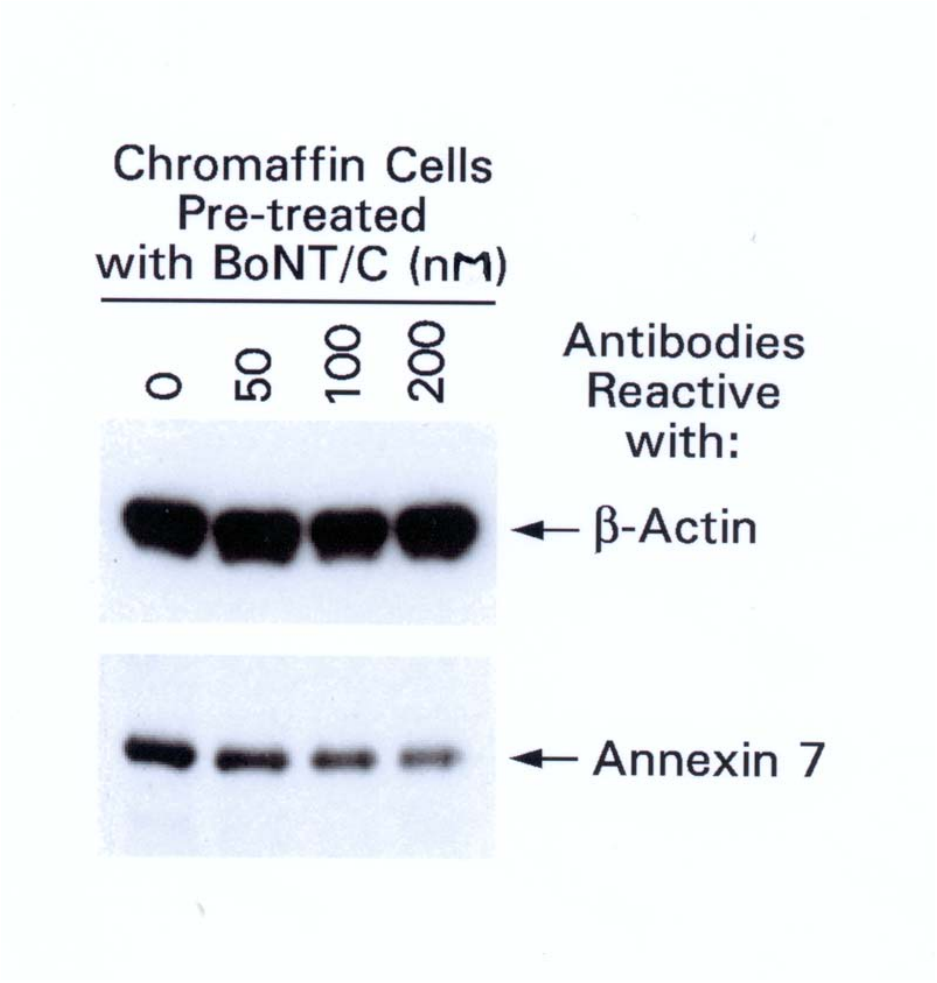

Figure 1. **Cleavage of ANX7 in permeabilized chromaffin cells by BoNT/C.** Equal amounts of protein from the lysates were subjected to SDS-PAGE and immunoblotting using antibodies against ANX7 and β-actin. The blot was developed using the ECL detection system (Pierce).

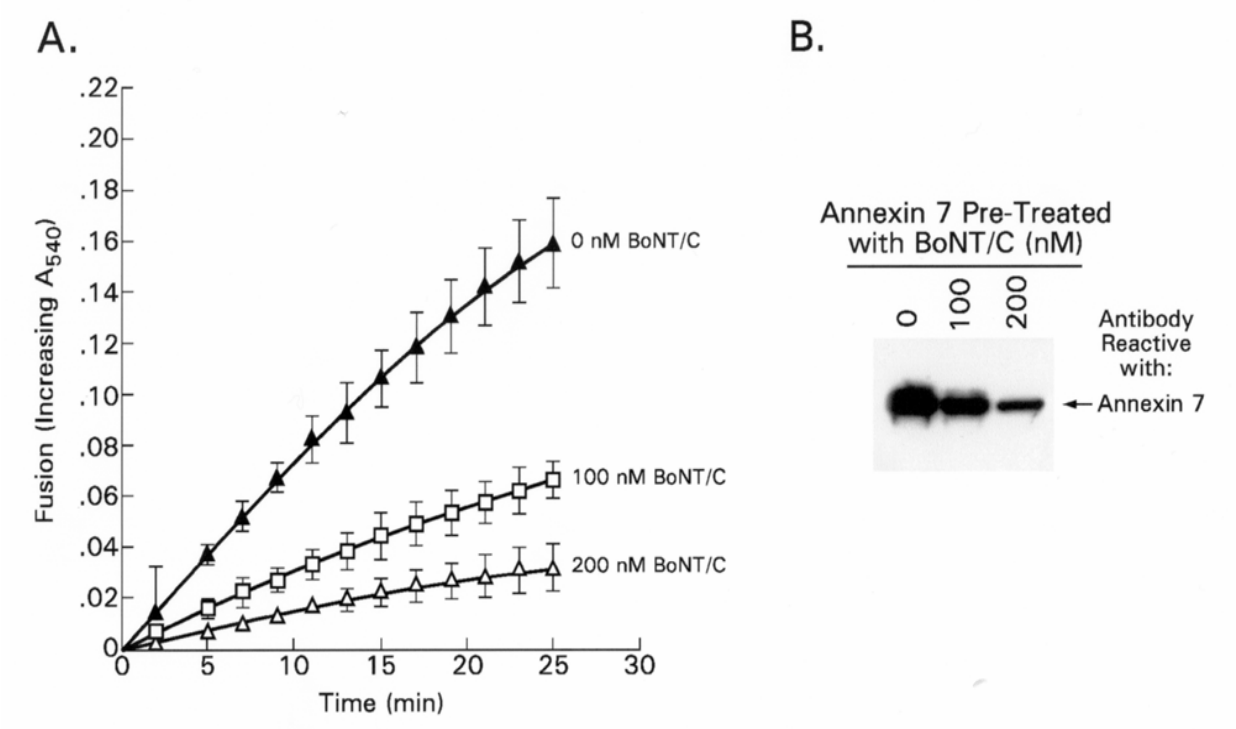

Figure 2. **Fusion of phosphatidylserine liposomes by BoNT/C-treated and untreated ANX7.** (***A***) ANX7, pretreated with or without BoNT/C, was added to a lipid vesicle fusion reaction. Fusion was measured at A_{540nm}. (***B***) At the end of each completed fusion experiment, the reactions were centrifuged, and the pellets were subjected to SDS-PAGE and immunoblotting.

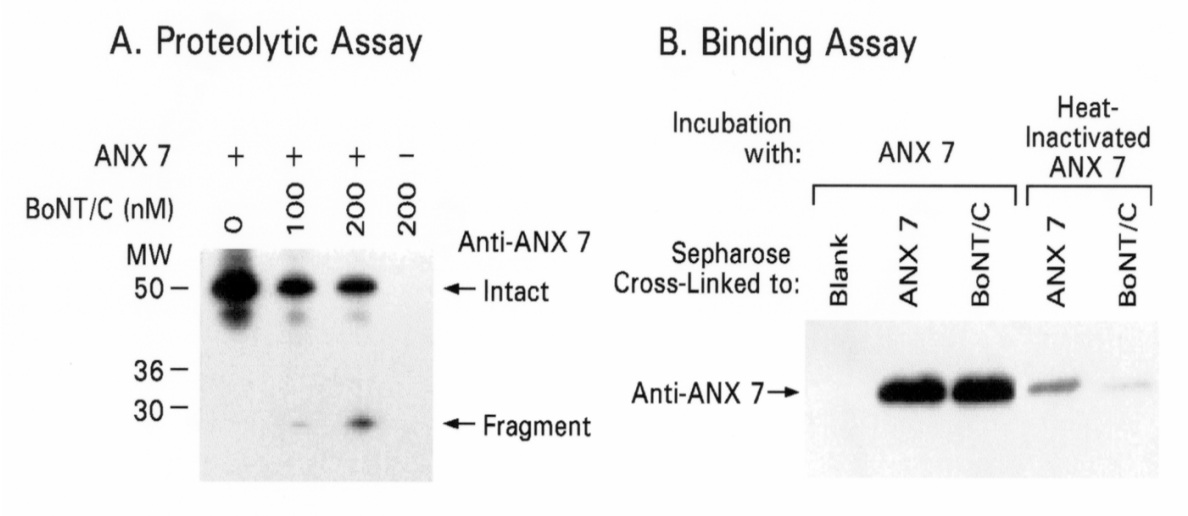

Figure 3. **BoNT/C cleaves recombinant ANX7 *in vitro.*** (***A***) ANX7 was incubated at 37^0C with or without BoNT/C, followed by SDS-PAGE and immunoblotting using an antibody against the N-terminal region of ANX7. The blot was developed using the SuperSignal Femto ECL detection system (Pierce). (***B***) ANX7 or heat-inactivated ANX7 were incubated at 4^0C with sepharose crosslinked to ANX7, BoNT/C, or nothing, followed by SDS-PAGE and immunoblotting.

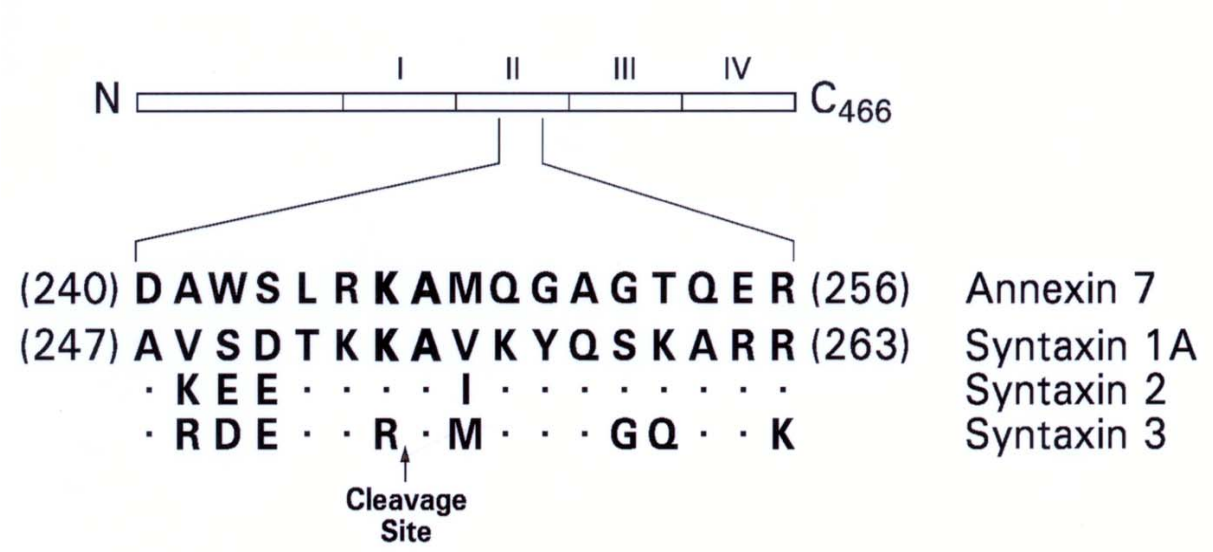

Figure 4. **Predicted cleavage site of BoNT/C on ANX7.** The portion of the amino acid sequence of ANX7 encompassing the predicted cleavage site of BoNT/C is enlarged and is compared with those of the syntaxin isoforms. *Numbers* indicate the corresponding positions of amino acids; *Roman numbers* indicate the positions of four annexin C-terminal repeats; and *dots* indicate residue conservation in the syntaxin family.

CHAPTER 5

Discussion

Guanosine triphosphate (GTP) and protein kinase C (PKC), in concert with calcium, are known to constitute a highly potent intracellular effector system for promoting exocytotic secretion from chromaffin cells and many other cell types (Barrowman *et al*., 1986; Howell *et al*., 1987; Haslam and Davidson, 1984; Luini and DeMatteis, 1990; Vallar *et al*., 1987; Oetting *et al*., 1986; Knight and Baker, 1985; Bittner *et al*., 1986; Bader *et al*., 1989; Ahner-Hilger *et al*., 1992; Pocotte *et al.*, 1985; Brocklehurst *et al*., 1985; TerBush and Holz, 1986; TerBush *et al*., 1988; Coorsen *et al*., 1990; Smolen *et al*., 1989; Smolen and Sandborg, 1990; Stojikovic *et al*., 1991; Persaud *et al*., 1989; Wollheim and Regazzi, 1990; Howell *et al*., 1990; Churcher and Gomperts, 1990; Sloan and Haslam, 1987; Walker and Watson, 1993; Cockcroft *et al*., 1987; Ahnert-Hilger *et al*., 1987; Lillie and Gomperts, 1991; Koopman and Jackson, 1990; Carroll *et al*., 1990). It has been proposed in the first model for exocytosis (Gomperts, 1990; Lillie and Gomperts, 1993), that these effectors might act through a common site, or on putative calcium and GTP target proteins that are closely associated with each other in the exocytotic machinery. The intense search for these mediators of exocytosis has led to the proposal of a more recent second exocytotic model, called the SNARE hypothesis, by Rothman and his colleagues (Rothman, 1994; Rothman and Orci, 1992**;** Sudhof, 1995**;** Chapman *et al*., 1995; Burgoyne and Morgan, 1998). The current version of the fusion machine hypothesis envisions a core complex formed between plasma membrane syntaxin and SNAP-25 and the synaptic vesicle protein synaptobrevin/VAMP, with vesicular synaptotagmin.

Synaptotagmin is identified as a low-affinity calcium sensor, which interacts with syntaxin. In addition, several members of the rab family of small GTP-binding proteins have been identified in mammalian and yeast as having a function in vesicle trafficking (Oberhauser *et al*., 1992). These proteins are also found to be associated with the SNARE fusion machine (White, 1992), suggesting that they might mediate the stimulatory action of GTP on exocytosis. However, with the exception of syntaxin, which is lethal, knockout mutations in the mouse for these genes (i.e. synaptotagmin and rab) have had little or no effect on the secretory process (Broadie *et al*., 1994; Geppert *et al*.,1994; 1997). Furthermore, none of these proteins presently identified in the hypothetical fusion machine actually fuse membranes (Rothman and Orci, 1992; Morgan, 1995; O'Connor *et al*., 1994; White, 1992). Therefore, the general opinion has been that the calcium sensor has yet to be discovered, and that other GTP-binding proteins, as yet unidentified, might control the activity of the fusion complex (Ferro-Novick and Jahn, 1994; O'Connor *et al*., 1994).

In the earlier model of exocytosis, Gomperts *et al*. (1990; 1993) predicted that the action of PKC in promoting exocytosis was to activate the putative calcium- and GTP-binding proteins at the docking/fusion site, and that subsequent phosphorylation by PKC triggered these proteins into mediating the exocytotic membrane fusion process. Several possible mediators of exocytosis, including both annexins 1 and 2 (Michner *et al*., 1986; Gould *et al*., 1986; Wang and Creutz, 1992; Johnstone *et al*., 1992; 1993), and SNAP-25 (Shimazaki *et al*., 1996), are found to be phosphorylated by PKC both *in vitro* and in secretory

cells. However, phosphorylation of these proteins by PKC markedly inhibits their functional activities, indicating that they are unlikely to mediate the positive action of PKC on exocytosis. Collectively, the specific sites of action for calcium, GTP and PKC in the fusion machine remain unknown.

Chromaffin cells and many other cell types contain a membrane fusion protein annexin 7 (ANX7), associated with secretory vesicles and plasma membranes (Kuijpers *et al*., 1992), which has an appropriate intrinsic K_d for calcium of approximately 200 μM (Creutz *et al*., 1978; Hong *et al*., 1982; Raynal and Pollard, 1994). In the early days of ANX7 research, when submicromolar levels of calcium were thought to drive secretion, it was sometimes conventional to dismiss the ANX7 K_d level as "too high" to be physiologically relevant to secretory processes. However, in view of the recent data showing that the local calcium concentration in the region immediately subadjacent to the exocytotic site is increased into the 50-300 μM range (see Chapter 1), the ANX7 K_d would now appear to be "just right".

The site of GTP action in exocytosis has been thought to be closely associated with the site of calcium action at some common site (Monck and Fernandez, 1994; Gomperts *et al*., 1987; Howell *et al*., 1987; Okano *et al*., 1993). Therefore, a good experimental goal would seem to be to search for a membrane fusion protein that is activated by both calcium and GTP. Parenthetically, Gomperts *et al*. (1987) predicted that the protein in question would also be activated by PKC.

For the question of how GTP acts to promote Ca^{+2}-dependent secretion, we have previously reported that ANX7 drives the Ca^{+2}-dependent membrane fusion reaction in a manner further activated by GTP (Caohuy *et al*., 1996). The mechanism of fusion activation depends on the unique ability of ANX7 to bind and hydrolyze GTP in a Ca^{+2}-dependent manner, both *in vitro* and in SLO-permeabilized chromaffin cells. Inasmuch as previous immunolocalization studies place ANX7 at exocytotic sites in chromaffin cells (Kuijpers *et al*., 1992), we have concluded that ANX7 may both detect and mediate the Ca^{+2}/GTP signal for exocytotic membrane fusion.

Together with the above observations, the present data clearly show that the protein in question in the Gomperts' exocytotic model is very likely to be ANX7. In Chapter 2, we show that ANX7 is phosphorylated by PKC, both *in vitro* and in secreting chromaffin cells. Similar to the GTP action on ANX7, PKC also significantly potentiates the ability of ANX7 to fuse lipid vesicles. More significantly, phosphorylation of ANX7 by PKC lowers the half-maximal concentration of calcium needed for this fusion process. The apparent K_d of calcium for phosphorylated ANX7 now becomes 50 μM, as opposed to that of 200 μM for the unphosphorylated form. These *in vitro* findings clearly show that activation of PKC triggers ANX7 to efficiently drive fusion of lipid vesicles, even at lower calcium concentrations. Remarkably, this condition appears to be physiologically relevant to that in the stimulus-secretion cascade. Indeed, the levels of PKC-dependent phosphorylation of endogenous ANX7 are closely correlated with the levels of catecholamine secretion from chromaffin cells in

response to stimulation of carbachol or nicotine. Thus, the positive action of PKC on exocytosis is indeed likely to be mediated by ANX7.

Furthermore, we report that GTP and PKC both mutually enhance the binding of each other to ANX7, and consequently potentiate further Ca^{+2}-dependent membrane fusion driven by ANX7 (see Chapter 3). These findings are remarkably comparable to the finding of activation of Ca^{+2}-dependent secretion by both GTPγS and PKC from secretory cells (Bader *et al*., 1989; Smolen and Sandborg, 1990; Sloan and Haslam, 1997; Cockcroft *et al*., 1987; Ahnert-Hilger *et al*., 1987; Lillie and Gomperts, 1991; Koopman and Jackson, 1990; Carroll *et al*., 1990). Furthermore, the *in vitro* data on ANX7 membrane fusion activity appear to be well correlated with what we have observed in the *in vivo* studies. In these *in vivo* studies with ANX7, the ratio of bound GDP/GTP and phosphorylation by PKC change in direct proportion to the extent of catecholamine release from [^{33}P]-labeled chromaffin cells in response to stimulation by carbachol, or to inhibition by various PKC inhibitors. This close correlation between the *in vivo* and the *in vitro* data thus implies that ANX7 functionally behaves like a G_E (G-protein for exocytosis; Lillie and Gomperts, 1993), and transduces the intracellular signals for exocytosis by simultaneously binding GTP and being phosphorylated by PKC in a Ca^{+2}-dependent manner.

In other *in vitro* assays, the efficiency of ANX7 phosphorylation by PKC is further enhanced by GTP and its non-hydrolyzable analogues, but not by GDPβS. Significantly, the concentrations of added guanine nucleotides that activate this ANX7 phosphorylation event are relevant to the physiological GTP

concentration range (Otero, 1990; Bourne *et al*., 1991). Reciprocally, the ANX7 phosphorylation by PKC substantially stimulates the basal levels of GTP binding and hydrolysis by ANX7. The significance of these results is that upon phosphorylation by PKC, the turnover number for ANX7 is now relatively equivalent to those of some known G-proteins, including EF-G, EF-Tu, tubulin, and the G components of adenylate cyclase and transducin, with turnover numbers of 0.012-0.25 mol/min/mol of protein (Chinali and Parmeggiania, 1980; Fasano *et al*., 1982; Carlier and Pantaloni, 1982; Brandt, 1983; Fung, 1983). Furthermore, the rate of GTP binding for phosphorylated ANX7 is quite similar to that of GTP hydrolysis, indicating that the hydrolytic/exchange reaction is rapid and is limited by GDP dissociation. Such an indication has been supported by the present data showing that the addition of excess GDPβS markedly inhibits the additive effect of PKC and GTPγS on ANX7 membrane fusion activity. This result strongly suggests that the exchange reaction of GDPβS for GTPγS is blocked by the excess molar concentration of GDPβS. Thus, it appears from these data that once ANX7-bound GTP is hydrolyzed, the newly formed GDP is released rapidly, and the empty nucleotide-binding pocket of ANX7 is ready to accommodate a new GTP molecule. ANX7 has a higher affinity for GTP than it has for GDP, thus indicating that the exchange is supported by energetic properties (Caohuy *et al*., 1996).

The present data show that phosphorylation of ANX7 by PKC is enhanced by GTP, and vice versa, that PKC-dependent phosphorylation of ANX7 substantially enhances the binding and hydrolysis of GTP by ANX7. Yet, the

mechanisms remain to be fully elucidated. Nonetheless, it is plausible to speculate that the ANX7 structural conformational change initially induced by calcium binding may be the turning step that leads to successful GTP binding to ANX7 and ANX7 phosphorylation by PKC. Indeed, these two processes, *in vitro*, are conditionally dependent on calcium. This fact suggests that calcium modifies the structural conformation of ANX7 sufficiently to permit efficient GTP binding (Caohuy *et al*.,1996) and enhanced labeling by PKC (see Fig. 2, Chapter 2). Subsequently, the combined actions of GTP and PKC on ANX7 may induce further change of this ANX7 conformation, and the newly formed ANX7 conformation may be instrumental for both PKC phosphorylation and the stimulated GTPase and rapid GDP/GTP exchange.

Based on these observations, ANX7 acts like a switch for membrane fusion (see Fig. 8, Chapter 3). On elevation of the intracellular calcium concentrations into the 50- to 200-μM range, ANX7 converts to the moderate fusogenic "on" state (ANX7-Ca^{+2}/Mg^{+2}-GDP), and simultaneously binds GTP to form the intermediate fusogenic state (ANX7-Ca^{+2}/Mg^{+2}-GTP). This process can be further potentiated by PKC. Conversely, subsequent GTP binding stimulates PKC phosphorylation of ANX7. These processes simultaneously transform ANX7 into a highly efficient Ca^{+2}-dependent membrane fusogen [**P**-(ANX7-Ca^{+2}/Mg^{+2}-GTP)]. Fusion then ensues until GTP is hydrolyzed, ANX7 phosphorylation is dephosphorylated and the calcium is reduced, leaving ANX7 again in the "off" state (ANX7-Mg^{+2}-GDP).

While certain other protein kinases, including PKA, PKG, and $pp60^{c\text{-}src}$, phosphorylate ANX7 efficiently, they do not substitute for PKC in potentiating GTP binding or membrane fusion (see Chapters 2 and 3). Thus, the lack of stimulation by these kinases on these ANX7 processes coincides with other observations showing that these kinases are not directly involved in regulated exocytosis (Bittner *et al*., 1986; Cheek and Burgoyne, 1987; Shono *et al*., 1997; Kumai *et al*., 1998; Ely *et al*., 1990; Ohnishi *et al*., 2001). In addition, the lack of effects of these phosphorylation events, compared to the consequences of PKC, serves as an important control for emphasizing the importance of PKC induced changes in ANX7 function.

Collectively, these observations on ANX7 are remarkably congruent with the original exocytotic model of Gomperts (1990). The ANX7 data are consistent with the concept that the stimulatory actions of calcium, GTP and PKC converge on ANX7 to drive membrane fusion occurring during exocytosis. To further support such an inference, we show that ANX7 is a non-SNARE proteolytic substrate for BoNT/C (see Chapter 4). BoNT/C, which is a zinc-dependent protease and a specific inhibitor of exocytosis (Dolly *et al*., 1994), efficiently cleaves ANX7 both *in vitro* and in permeabilized chromaffin cells. This proteolytic activity is concurrent with BoNT/C-dependent inhibition of ANX7 membrane fusion activity *in vitro*, and with inhibition of catecholamine secretion *in vivo*, respectively. These findings significantly parallel the proteolytic effect of this toxin on syntaxin (Schiavo *et al*., 1995) and SNAP-25 (Blasi *et al*., 1993), which are protein components of the SNARE hypothesis (Rothman and Orci, 1992).

Inasmuch as the identification of SNARE proteins as targets for BoNTs has been taken as *prima facie* evidence favoring the SNARE hypothesis for exocytotic membrane fusion, the apparent role of ANX7 in the exocytotic membrane fusion process thus cannot be excluded. This conclusion is further supported by our recent report that a nullizygous (-/-) knockout of the anx7 gene in mouse is lethal, and that insulin secretion from islets of Langerhans of the heterozygous knockout anx7 (+/-) mouse is defective (Srivastava *et al*., 1999).

It is true that ANX7 alone is able to mediate efficiently both membrane contact and fusion reactions *in vitro* (Nir et al., 1987; Pollard et al., 1991). However, the exocytotic process is likely to be much more complicated. Therefore, although the fusion reaction, *per se*, may be mediated by ANX7 or related molecules, *in vivo*, it remains likely that additional contributions to specific docking processes may be derived from other protein components in the cell. We do not exclude the possibility that these hypothetical protein components may include those specified by the SNARE hypothesis. In this respect, our lab has found that ANX7 indeed interacts with syntaxin, a SNARE protein, as indicated by both the co-immunoprecipitation techniques and the yeast two-hybrid system (Srivastava, Jozwik, Nachson, and Pollard; unpublished observations). This finding thus has led us to propose a new version of the exocytotic model (Fig. 1). In this model, the SNARE complex at the docking/ fusion site now contains several ANX7 molecules, one of which interacts directly with syntaxin, a SNARE protein. These ANX7 molecules then undergo conformational changes induced by calcium, GTP and PKC that expose their

hydrophobic domains and eventually promote polymerization of ANX7 molecules. Indeed, it has been shown by light scattering and electron microscopy that the Ca^{+2}-activated form of ANX7 has a polymeric structure, and these polymers themselves can form higher order polymers by side-to-side association (Creutz *et al*., 1979). Finally, along with the disassembly of the SNARE complex, these ANX7 polymers then form a hydrophobic bridge that links two adjacent target membranes and eventually causes the two membranes to fuse.

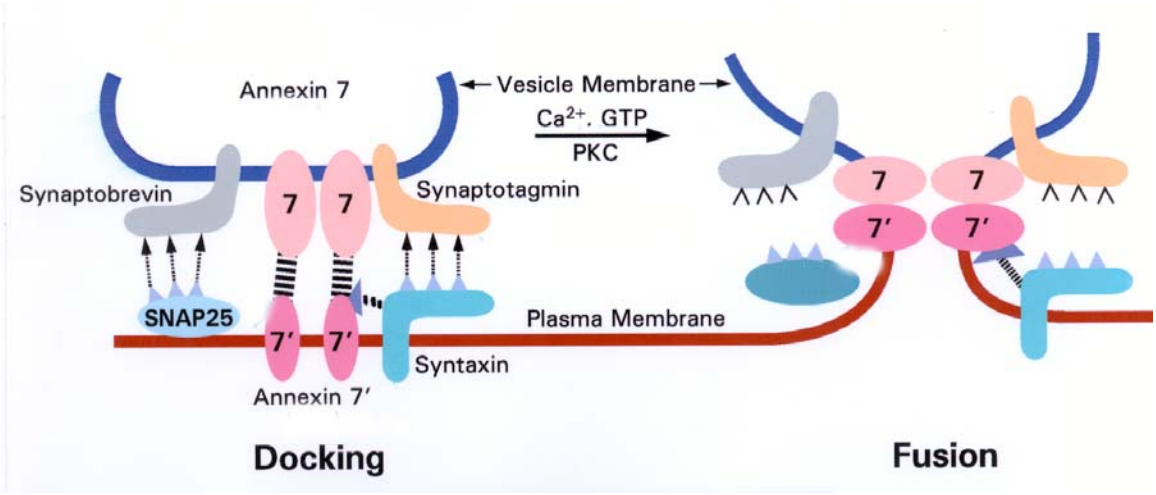

Figure 1. **Model for annexin 7-driven exocytotic membrane fusion.** The plasma protein syntaxin of the SNARE complex, which includes synaptobrevin, synaptotagmin and SNAP-25, is the binding target of ANX7. ANX7 in turn recruits several other ANX7 molecules, and all of these ANX7 molecules undergo conformational changes induced by calcium, GTP and PKC. These activated ANX7 molecules then polymerize and consequently cause fusion of the vesicle membrane with the inner surface of the plasma membrane. *Broken arrows* indicate the protein-protein interaction.

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